Deciphering the impact of glycosylation alterations in activity and function of γδT cells in autoimmunity

Beatriz Santos Pereira

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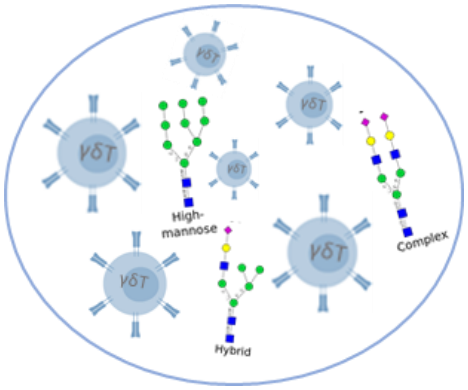

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Beatriz Santos Pereira
Mestrado em Biologia Celular e Molecular
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2020

Orientador

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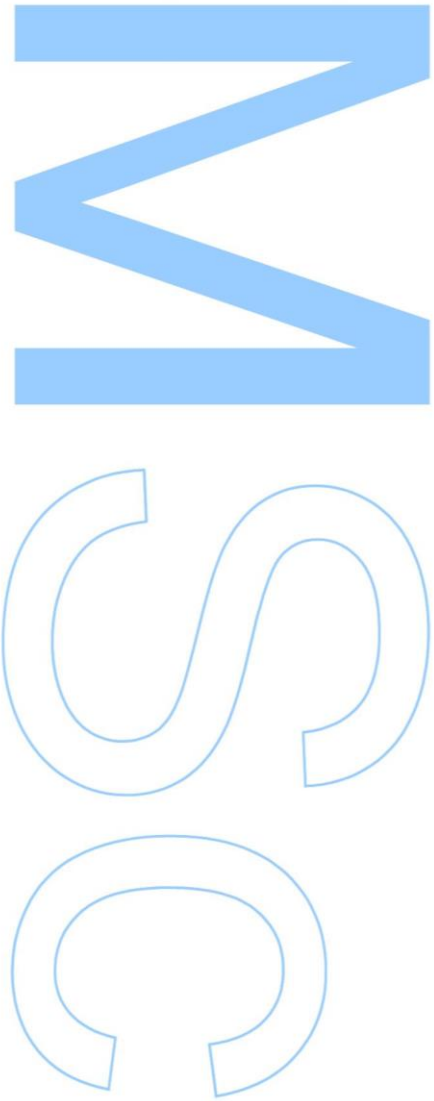

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Eu, Beatriz Santos Pereira, aluna com o número 201504925 do mestrado de Biologia Celular e Molecular da edição de 2018/2020, declaro por minha honra que sou a autora da totalidade do texto apresentado, não apresento texto plagiado, e tomei conhecimento das consequências de uma situação de plágio.

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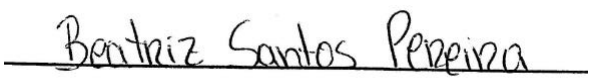

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Abstract

Systemic lupus erythematosus (SLE) is a chronic and debilitating autoimmune disease, prevalent in women, characterized mainly by a massive antibody production and immune complexes accumulation, in which lupus nephritis is one of the most severe complications. The loss of immunological tolerance is involved as a trigger for SLE, although the exact etiophatogenesis is still not fully elucidated. Glycosylation is a major protein post-translational modification involved in the attachment of carbohydrates (glycans) to other saccharides, proteins or lipids, by the action of specific enzymes. Glycans and glycan-binding proteins contribute to a variety of important biological functions and are crucial components in the correct recognition of pathogens or damage cells by the immune cells, thus glycosylation acts as a master regulator of the immune system. Changes in protein glycosylation are known to interfere with both innate and adaptive immune responses. The γδT cells are a small population of T lymphocytes, which are implicated in the immunopathogenesis of autoimmune diseases. Since glycosylation interferes with T cell biology, we herein hypothesize whether changes in the glycosylation profile of γδT cells determine its activity and functions associated with SLE immunopathogenesis. In this work, we aim to unravel the impact of glycosylation alterations in γδT cells' function and immune response associated with SLE pathogenesis, focusing on the potential of glyco-modulation as a specific targeted-therapy for SLE patients. Firstly, we characterized γδT cells glycosylation profile in kidney clinical samples from SLE patients and in an autoimmune disease-susceptible mouse model. We then, modulated the γδT cells glycosylation aiming to access the impact in γδT cells activity and functions. Our results demonstrated a clear γδT cells infiltration in the kidney parenchyma of SLE patients, as well as in organs of autoimmune disease-susceptible mouse model. We showed an altered glycosylation profile of peripheral blood γδT cells from SLE patients, characterized by an increased high-mannose *N*-glycans expression. Interestingly, mouse model lacking β1,6-GlcNAc branched *N*-glycans showed an altered γδT cells' glycosylation profile associated with a tissue-specific distribution and cell response. Furthermore, we demonstrated that modulating γδT cells glycosylation gave an effect in the regulation of immune response in which the block of β1,6-GlcNAc branched *N*-glycans led to an autoreactive and pro-inflammatory status of these cells. Lastly, we demonstrated that γδT cells express glycan-binding receptors at the cell surface and the lack of branched *N*-glycans in γδT cells increases the expression of specific C-type lectins receptors at the cell surface.

Altogether, we described for the first time an abnormal *N*-glycosylation of γδT cells implicated in SLE pathogenesis, proposing the β1,6-GlcNac branched *N*-glycosylation pathway as an important glycan moiety implicated in the modulation of γδT cells response in SLE, as well as a potential targeted mechanism to control SLE development and progression.

Key words: Systemic lupus erythematosus; Autoimmunity; *N*-glycosylation; β1,6-GlcNAc branched *N*-glycans; γδT cells; C-type lectins

Resumo

O lúpus eritematoso sistêmico (SLE) é uma doença autoimune crônica e debilitante, prevalente em mulheres, principalmente caracterizada por uma excessiva produção de anticorpos e acumulação de complexos imunes, sendo a nefrite lúpica (LN) uma das complicações mais severas. A perda da tolerância imunológica está envolvida como um estímulo em SLE, contudo a exata etiopatogénese ainda não está totalmente elucidada. A glicosilação é um mecanismo pós-tradução envolvido na adição de carboidratos (glicanos) a outros sacarídeos, proteínas ou lípidos, pela ação de enzimas específicas. Os glicanos e as proteínas onde estes se ligam contribuem para uma variedade de funções biológicas importantes e são componentes cruciais no correto reconhecimento de agentes patogénicos ou de células danificadas pelas células imunes e por isso a glicosilação atua na regulação do sistema imune. Alterações na glicosilação proteica são conhecidas por interferir na resposta do sistema immune inato e adaptativo. As células T γδ são uma pequena população de linfócitos T, que estão envolvidos na imunopatogénese de doenças autoimunes. Como a glicosilação interfere com a biologia das células T, a nossa hipótese é que as alterações no perfil de glicosilação das células T γδ determinam a sua atividade e função e de que forma estão associadas à imunopatogénese de SLE. Neste trabalho, pretendemos clarificar o impacto das alterações da glicosilação na função e na resposta imune das células T γδ associadas com a imunopatogénese de SLE, destacando o potencial da glico-modulação como uma terapia-alvo específica para pacientes com SLE. Primeiramente, foi caracterizado o perfil de glicosilação das células T γδ de amostras clínicas de rim dos pacientes e ainda de um modelo animal suscetível a doenças do tipo autoimunes. Posteriormente, modulamos a glicosilação das células T γδ com o objetivo de verificar o impacto na atividade e função destas células. Os nossos resultados demonstraram uma clara infiltração das células T γδ no parênquima renal de pacientes com SLE, assim como outros órgãos de um modelo de ratinho suscetível a doenças autoimunes. Também demonstramos uma alteração do perfil de glicosilação das células T γδ do sangue periférico dos doentes com SLE, caracterizada por um aumento de *N*-glicanos manosilados. No modelo animal sem *N*-glicanos ramificados verificou-se uma alteração do perfil de glicosilação das células T γδ associada a uma resposta celular e distribuição especifica em cada tecido. Para além disso, demonstramos que modulando a glicosilação das células T γδ ocorre uma regulação da resposta imune, no qual o bloqueio dos glicanos ramificados levou a um estado autoreativo e pro-inflamatório

nestas células. Por último, demonstramos que as células T γδ expressam recetores de glicanos na superfície celular e que a diminuição dos *N*-glicanos ramificados nestas células aumenta a expressão dos recetores (lectinas do tipo C) na superfície das mesmas. Concluindo, descrevemos pela primeira vez que as alterações da *N*-glicosilação nas células T γδ estão implicadas na patogénese de SLE, propondo os glicanos ramificados como estruturas envolvidas na modulação da resposta das células T γδ em SLE, assim como um potencial mecanismo específico para controlar o desenvolvimento e a progressão de SLE.

Palavras-chave: Lúpus eritematoso sistémico, autoimunidade, *N*-glicosilação; *N*-glicanos ramificados; células T γδ; lectinas do tipo C

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Figure 17 – Cytokines in mesenteric lymph nodes (MLNs) and kidney from 15 months-old *Mgat5*$^{-/-}$ (KO) mice compared with WT mice. A) Fold-change of the MFI of intracellular IL-17 and IL-22 in γδT cells, after PMA/Ion stimulation of γδT cells from the MLNs of *Mgat5*-aging mice, showing decreased expression of both cytokines in γδT cells from the KO compared with WT mice (n=5 WT, n=5 KO, the outliers in each analysis were removed). The results were normalized to the mean value of MFI from WT mice. Each dot represents one individual biological sample, presented as mean ± SD. **B)** Concentration of IL-17A and IFNγ in the supernatants of renal explants from *Mgat5*-aging mice, expressed as pg/mL/mg of tissue, (n=5 WT, n=5 KO). Showing a slight increase in IL-17A and IFNγ in the KO compared with WT mice. Bars represent mean ± SD. Statistical significance was assessed by t- test with Mann-Whitney test, although both analysis do not reveal any significance statistical (significant *p*-value ≤ 0.05). 39

Figure 18 – *In vitro* re-program of γδT cells glycosylation profile: Optimization assay to select the ideal culture time and activation marker for *in vitro* ablation of complex *N*-glycans, through flow cytometry. A) Activation profile (MFI) of KF-treated γδT cells (KF) and non-treated cells (basal) evaluated with different activation markers - CD25, HLA-DR and CD69 - at different timepoints (7 h, 24 h, 48 h and 72 h). The results showed increased activation in all timepoints of KF-treated γδT cells by CD25 and HLA-DR reactivity, although CD69 overexpression was only observed at 48 h. **B)** Glycosylation profile (MFI) of KF-treated γδT cells (KF) and non-treated cells (basal), at different timepoints (7 h, 24 h, 48 h and 72 h). The results showed increased expression of high-mannose *N*-glycans (by GNA binding) and decreased expression of branched *N*-glycans (by L-PHA binding) of KF-treated γδT cells, in the last three timepoints. The selected activation marker and culture time were CD25 and 24 h, respectively. The results are presented as mean ± SD. (Basal n=1; KF n=1, technical duplicates) Statistical analysis was not assessed since the number of samples is very few. 41

Figure 19 – Activation, glycosylation and cytokine profile of KF-treated γδT cells compared with non-treated cells, assessed by flow cytometry. A) Activation and glycosylation profile (MFI) of KF-treated γδT cells (KF cells) and non-treated cells (basal cells). It is showed enhanced activation of KF cells (CD25), a decreased of branched *N*-glycans (L-PHA) and α-2,6 sialic acid (SNA), as well as an increase of high-mannose *N*-glycans (GNA staining). **B)** Cytokine profile (MFI) of KF-treated γδT cells (KF cells) and non-treated cells (basal). The results showed an increased expression of pro-inflammatory cytokines (IL-17 and IFNγ) and a decreased expression of IL-10 cytokine. The percentage of IL-17- and IFNγ-producing γδT cells (γδ17T and γδIFN$_\gamma$T cells) was higher in KF cells, and the opposite for IL-10-producing γδT cells (γδ10T cells). Each dot

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Abbreviations

ACK	Ammonium Chloride Potassium
APC	Antigen-Presenting Cell
Asn	Asparagine
BAFF	B Cell-Activating Factor
BSA	Bovine Serum Albumin
CHP/HSA	Porto Centre Hospital-Hospital Santo Antonio
CHSJ	São João University Hospital
CHUC	Coimbra University Hospital
cKO	Conditional Knock-Out
CTLA-4	Cytotoxic T-Lymphocyte Protein 4
CTL	C-type Lectin
DAMP	Damage-Associated Molecular Pattern
DC-SIGN	DC-Specific Intercellular Adhesion Molecule-3-Grabbing Non-Integrin
DC	Dendritic Cell
Dectin	Dendritic Cell-Associated C-Type Lectin
Dol-P	Dolichol Phosphate
EAE	Experimental Autoimmune Encephalomyelitis
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum Associated Degradation
EULAR	European League Against Rheumatism
FACS	Fluorescent-Activated Cell Sorting
FBS	Fetal Bovine Serum
FFPE	Formalin-Fixed Paraffin-Embedded
FVD	Fixable Viability Dye
GA	Golgi Apparatus
Gal	Galectin
GBP	Glycan-Binding Protein
Glc	Glucose
GlcNAc	*N*-Acetylglucosamine
GNA	*Galanthus Nivalis* Lectin
GnT	*N*-Acetylglucosaminyltransferase
GPI	Glycosylphosphatidylinositol
Rag1	Recombination Activating 1
HBSS	Hanks' Balanced Salt Solution
HLA-DR	Major Histocompatibility Complex-II Cell Surface Receptor
HRP	Horseradish Peroxidase
IBD	Inflammatory Bowel Disease
IFN	Interferon
IgG	Immunoglobin G
IL	Interleukin
KF	Kifunensine
KO	Knock-Out
L-PHA	Phaseolus Vulgaris Leucoagglutinin Lectin
LEL	Lycopersicon Esculentum Lectin
LN	Lupus Nephritis
mAb	Monoclonal Antibody
Man	Mannose
MAN1A	Mannosidase alfa class 1A

MAN2A	Mannosidase alfa class 2A
MBL	Mannose-Binding Protein
MFI	Median Fluorescence Intensity
MGAT	Mannosidase Acetylglucosaminyltransferase
MGL	Macrophage Galactose Lectin
MHC	Major Histocompatibility Complex
Mincle	Macrophage Inducible C-Type Lectin
MR	Mannose Receptor
MS	Multiple Sclerosis
NET	Neutrophil Extracellular Trap
NK	Natural Killer
OST	Oligosaccharyltransferase
OVA	Ovalbumin
PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffer Saline
PBST	Phosphate Buffer Saline With 0.05% Tween20
Pen-Strep	Penicillin-Streptomycin
Poly-LacNAc	Poly-*N*-Acetyllactosamine
PRR	Pattern Recognition Receptor
RA	Rheumatoid Arthritis
Rag1	Recombination Activating 1
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
SDS-PAGE	Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis
Ser	Serine
SLEDAI	Systemic Lupus Erythematosus Disease Activity Index
SLE LN	Systemic Lupus Erythematosus with Lupus Nephritis
SLE nLN	Systemic Lupus Erythematosus without Lupus Nephritis
SNA	Sambucus Nigra Lectin
TCR	T Cell Receptor
Th	Helper T Cells
Thr	Threonine
TNF-α	Tumour Necrosis Factor-α
Treg	Regulatory T Cells
UC	Ulcerative Colitis
UniProt	Universal Protein Resource
WT	Wild-Type
α-Man	α1-2 Mannosidase
γδIFN$_\gamma$T cells	IFN$_\gamma$-producing γδT cells
γδ10T cells	IL-10-producing γδT cells
γδ17T cells	IL-17-producing γδT cells

Introduction

1. Lupus

Lupus is a multisystemic and autoimmune disease which is divided into four types: neonatal lupus erythematosus which results in maternal autoantibodies acting against the new-born; cutaneous lupus erythematosus, a chronic skin manifestation; drug-induced lupus triggered by certain drugs; and systemic lupus erythematosus, the most common form of lupus associated with severe complications[1–3].

1.1 Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a chronic, relapsing and incurable autoimmune disease that affects mostly women, nine times more frequently than men, between the ages of 16-55 years-old[4,5]. The incidence and prevalence rates of SLE differ worldwide with a range of 0.3-23 per 100 000 persons per year for incidence and 20-241 per 100 000 persons for prevalence (Figure 1)[6,7]. It is also important to note that these rates differ with sex, age, and ethnicity. For example, in SLE, is observed a higher incidence and prevalence rate in people with African and Asian background than in the remaining population[7]. Despite the improvements in early diagnosis and treatment, as well as in survival rates of 80%-92% at 10 years, this complex disease is still very debilitating[8,9]. Most SLE patients deal with organ failure, infections, or cardiovascular complications, which has been reported as premature death causes compared to the general population[10].

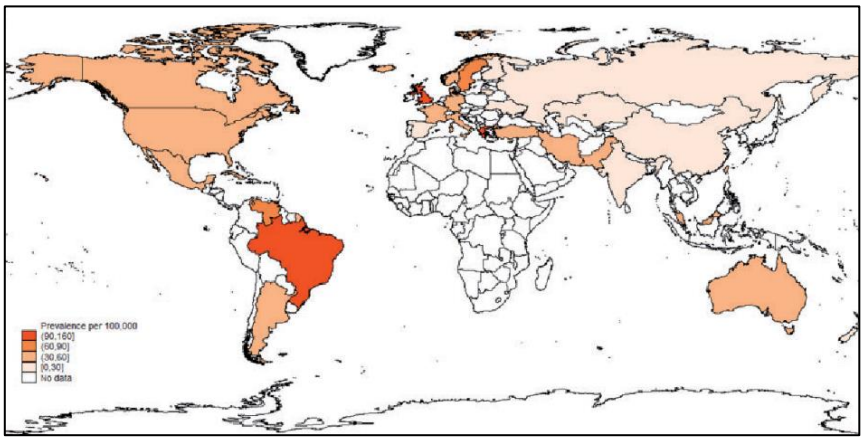

Figure 1 – The worldwide prevalence of Systemic Lupus Erythematosus (SLE), based on epidemiological studies. The prevalence of SLE differ with sex, age and ethnicity and despite the lack of studies in Africa, people with African and Asian background have the highest incidence and prevalence levels. Adapted from Rees *et* al., 2017[6].

The etiology of SLE is still unknown, however, some evidence shows genetic (e.g. epigenetic studies), hormonal, environmental (e.g. ultraviolet light, vitamin D, drugs, infections) and immune factors as important triggers associated with the initiation and progression of this complex disease, or even the combination of different factors[11]. Additionally, genome-wide association studies using single-nucleotide polymorphisms showed the presence of genomic regions in SLE patients which could indicate a predisposition to developing SLE[12–14]. Women are the most affected by SLE and start to develop this disease between puberty and menopause when sex hormones play an important role during these phases. Therefore, there are some studies that not only correlate some hormones directly with SLE susceptibility but also suggest a therapy based on hormonal modulation[15–18]. Several studies show a deficit in vitamin D as a risk factor to developed and aggravate SLE and some of them suggest supplementation with vitamin D in prevention and control of this disease[19–23]. Patients with SLE show significantly low levels of vitamin D due to avoidance of sunlight exposure and therapeutic approaches which modify the metabolism of this vitamin[22].

In the pathogenesis of SLE a break of self-tolerance occurs, along with an immune dysregulation of both innate and adaptive immune systems (Figure 3). The accumulation of nucleic acids, released by apoptotic cells, leads to a massive production of autoantibodies that bind to nucleic acids resulting in the formation of immune complexes that cause tissue damage[24,25]. Consequently, these cellular alterations are reflected in a wide spectrum of clinical manifestations, that range from skin rashes, mouth and nose ulcers, pain, swollen joints, heart and lung inflammation and renal complications (Figure 3)[26]. SLE is a very heterogeneous disease characterized by unpredictable flares that alternate with periods of disease remission. In 1971, the American Rheumatism Association published the first report about the criteria for the diagnosis and classification of SLE[27]. In 1997 the classification criteria was updated by *Hochberg* and currently used[28]. However, the European League Against Rheumatism (EULAR) and the American College of Rheumatology collected all the new findings of this disease and developed an updated classification criteria for SLE patients, where a total score is given by each criteria score (Figure 2)[29]. In summary, if the total score reaches at least 10 points, the individual is diagnosed with SLE. After diagnosis, it is important to access the disease activity index and tissue damage index to evaluate the severity of the disease and monitor the therapy of each patient. One of the biggest problems with the disease activity index is the heterogeneity of validated index worldwide, for example, European Consensus Lupus Activity Measure, Systemic Lupus Activity Measure; and SLE Disease Activity Index (SLEDAI)[30–32]. The most used index in

SLE are: SLEDAI and Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index for SLE[33]. SLEDAI takes into consideration 9 parameters based on organ system, such as central nervous system, vascular, renal, musculoskeletal, serosal, dermal, immunologic, constitutional and hematologic, where is assigned a score to each parameter and the total score gives the SLE activity index[34]. This quantitative index could be used to define a qualitative SLE activity, e.g. remission, low, mild, moderate, or severe disease activity.

Entry criterion
Antinuclear antibodies (ANA) at a titer of ≥1:80 on Hep-2 cells or an equivalent positive test (ever)

↓

If absent, do not classify as SLE
If present, apply additive criteria

↓

Clinical domains and criteria	Weight	Immunology domains and criteria	Weight
Constitutional		***Antiphospholipid antibodies***	
Fever	2	Anti-cardiolipin antibodies OR	
Hematologic		Anti-β2GP1 antibodies OR	
Leukopenia	3	Lupus anticoagulant	2
Thrombocytopenia	4	***Complement proteins***	
Autoimmune hemolysis	4	Low C3 OR low C4	3
Neuropsychiatric		Low C3 AND low C4	4
Delirium	2	***SLE-specific antibodies***	
Psychosis	3	Anti-ds DNA antibody* OR	
Seizure	5	Anti-Smith antibody	6
Mucocutaneous			
Non-scarring alopecia	2		
Oral ulcers	2		
Subacute cutaneous OR discoid lupus	4		
Acute cutaneous lupus	6		
Serosal			
Pleural or pericardial effusion	5		
Acute pericarditis	6		
Musculoskeletal			
Joint involvement	6		
Renal			
Proteinuria >0.5g/24h	4		
Renal biopsy Class II or V lupus nephritis	8		
Renal biopsy Class III or IV lupus nephritis	10		
Total score:			

↓

Classify as Systemic Lupus Erythematosus with a score of 10 or more if entry criterion fulfilled.

Figure 2 – European League Against Rheumatism/American College of Rheumatology classification criteria for Systemic Lupus Erythematosus. *Note: In an assay with at least 90% specificity against relevant disease controls. Adapted from Aringer *et* al. 2019[29].

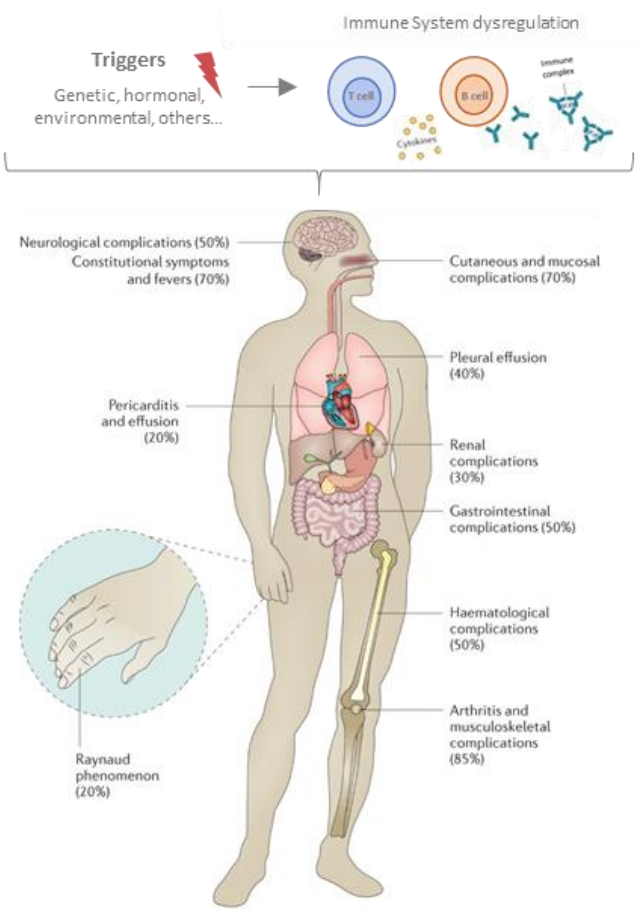

Figure 3 - Immune dysregulation and clinical manifestations of SLE. The etiology of SLE is still unknown, although genetic, hormonal, environmental and immune factors are important triggers associated with the initiation and progression of this disease. In the immunopathogenesis of SLE occurs an immune dysregulation of innate and adaptive immune systems, illustrated as cytokines release, accumulation of nucleic acids and massive production of autoantibodies that bind to nucleic acids resulting in the formation of immune complexes. Consequently, is reflected in a wide spectrum of clinical manifestations that affect different organ systems. Adapted from Kaul *et* al., 2016[26].

SLE remains an incurable disease and the current clinical management attempts to control the disease activity by managing the flares, preventing tissue and organ damage and aiming to preserve the quality of life of each patient[35]. The therapies to manage SLE are based in antimalarial drugs (hydroxychloroquine) and glucocorticoids. If patients do not respond well to the first line of treatment, a step-up approach to immunomodulating/immunosuppressive agents, such as methotrexate, azathioprine, mycophenolate, or cyclophosphamide is implemented. Finally, if the patients are unresponsive to the standard therapy, they start cycles with biologic drugs, such as belimumab (anti-B cell activating factor; anti-BAFF) and rituximab (anti-CD20), to control the disease progression[36,37]. Despite effective, this line of treatment presents high costs, as well as long-term secondary effects, becoming unsuitable to be used as a regular therapy. Although, novel SLE therapies arose in the last years, a lot of complications and side effects exist, such as toxicity, increased susceptibility to infections, risk of

cardiovascular disease and the disease is still incurable[35,38,39]. Therefore, it is crucial to further understand the molecular mechanisms underlying the development of SLE, envisioning the development of more specific and sensitive diagnostic and prognostic biomarkers, as well as novel targets for the design of optimized treatments that avoid therapy noxious side effects.

1.1.1 Lupus nephritis

Lupus nephritis (LN) is a kidney disorder and one of the most severe complication of SLE. The LN is initiated by the accumulation and deposition of immune complexes, associated with activation of the complement system leading to inflammation and tissue/organ damage, such as renal failure[40]. The frequency of LN differs from race/ethnicity and clinical presentation, however in general consensus up to 40% of SLE patients will develop kidney disease[41–43]. Furthermore, this devastating renal manifestation presents high morbidity and mortality and the diagnosis is still based on renal biopsy[44,45]. The treatments aim to ensure patient survival, preventing chronic kidney disease and improving quality of life, by the administration of immunosuppressive agents in combination with glucocorticoids[45]. However, when total kidney function is lost, the remaining alternative is dialysis and/or renal transplantation.

2. Immune system and SLE

The immune system is present in all vertebrates and involves homeostatic mechanisms capable of immune tolerance and host defence. It is divided into two major interconnected subsystems: the innate and adaptive immune systems that protect the body against foreign substances, such as pathogens (virus, parasites, fungi, and bacteria), non-pathological compounds or toxic molecules and/or damaged cells[46]. *Peter Medawar* showed the presence of "acquired immunological tolerance", a state of unresponsiveness of the immune system against a substance that has the ability to produce an immune response[47]. This mechanism allows the immune system to discriminate the self from non-self, by eliminating foreign antigens and avoiding an immune response against self-antigens. The tolerance against self-antigens, known as self-tolerance, it is only possible by the inactivation or elimination of self-reactive cells[48]. However, aberrant self-tolerance results in loss of homeostasis, leading to autoimmune diseases, like SLE and rheumatoid arthritis (RA), as it is described below.

2.1 Innate and adaptive immune responses

The innate immunity (or natural immunity) is a non-specific immune response and the first line of defence after an invasion by pathogens or other substances[46]. In contrast, the adaptive immunity (or acquired immunity) is a late antigen-specific response that allows the creation of immunological memory and is divided into humoral immunity and cell-mediated immunity. The components of innate immunity include: (1) cellular and chemical barriers, such as, skin, antimicrobial products, epithelia, and mucus, (2) blood proteins, such as proteins of the complement system and some lectins, and finally (3) cellular components, such as a) **phagocytic cells** which include neutrophils and macrophages that are responsible for the ingestion and destruction of pathogens with a role in inflammation; b) **dendritic cells (DCs)** which are the link between the innate and adaptive immunity and the most specialized antigen-presenting cells; c) **mast cells**; d) **natural killer (NK) cells** that have cytotoxic effector functions; and e) **innate lymphoid cells** (Figure 4). Whereas, the components of adaptive immunity are mainly lymphocytes, such as B and T lymphocytes and specific molecules (Figure 4).

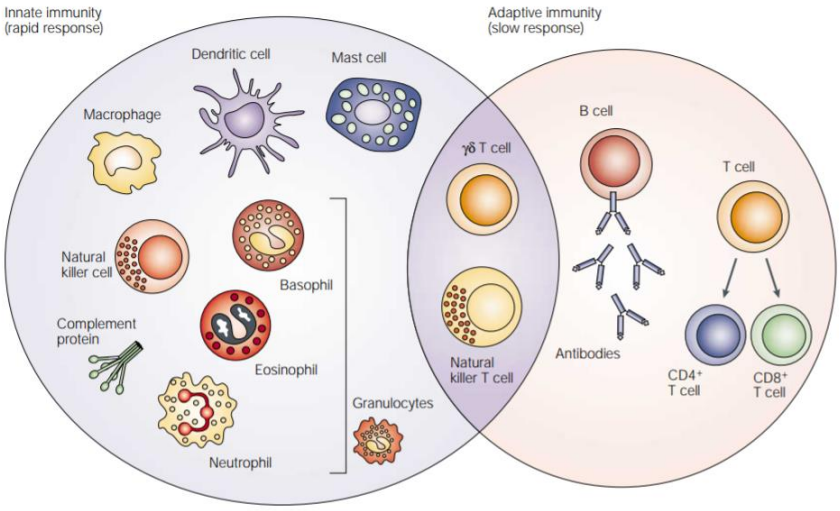

Figure 4 – The components of innate and adaptive immunity. The innate immune system is characterized by a rapid response, where mast cells, dendritic cells and phagocytic cells are the principal components. The adaptive immunity is described as a slow response, that comprise B and T lymphocytes. Both natural killer and γδT cells are in the "border" between innate and adaptive immunity[49].

DCs are specialized antigen-presenting cells (APCs) that play a crucial link between the innate and adaptive immune systems and are responsible for sensing pathogen-associated molecular patterns (PAMPs) and present them to naïve T lymphocytes. DCs express two classes of major histocompatibility complex (MHC) surface molecules, the class I (MHC-I) and II (MHC-II) which are essential for peptides presentation to specific subsets of T cells[50]. Therefore, after a pathogen invasion, immature resident DCs capture the host-antigens, become mature and migrate to lymphoid organs where present the antigens to T cells through MHC molecules, leading to T cell activation[51]. It is important to refer that B cells are also APCs and are similarly enrolled in T cell activation, for example by presenting antigens on MHC molecules to T cells[52–54]. Additionally, activated B cells can differentiate into plasma B cells which are able of antibody-production, or memory B cells essentials for dealing rapid response against future pathogen invasions[55,56].

The human body comprises protective systems capable of recognition and elimination of a huge array of pathogens and damaged cells, which could cause extensive damages in our tissues. One of these mechanisms is a process called inflammation and is initiated by APCs, which includes mainly DCs, macrophages, mast cells, NK cells, and B lymphocytes. APCs express pattern recognition receptors (PRRs), e.g. C-type lectin receptors, that allow the recognition of conserved PAMPs, such as nucleic acids, carbohydrates, lipids, and proteins, as well as damage-associated molecular patterns (DAMPs) released by damaged/stressed cells to its surface[57,58]. This recognition trigger other immune responses, for example, signalling pathways or

cytokines, promoting the process of inflammation and stimulating adaptive immune responses[58–60]. Monocytes are long-live cells that migrate to the infected tissue and differentiate into tissue-resident macrophages or immature DCs. Following, macrophages and neutrophils produce antimicrobial compounds, like reactive oxygen species, nitric oxide, and proteolytic enzymes, creating an environment that helps to kill pathogens[61]. Furthermore, neutrophils, macrophages and immature DCs present phagocytic activity, along with pro-inflammatory functions through the secretion of large amounts of pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6 and tumour necrosis factor-α (TNF-α)[62]. In response to environmental cytokines and other compounds produced by mast cells (e.g. histamine) vascular dilation occurs, as well as increased permeability and blood flow. Consequently, more leukocytes and blood proteins (complement proteins, antibodies) are accumulated in the infected area, leading to the manifestation of systemic effects including fever, redness, swelling and pain. Equally important, some cytokines also play a role in preventing viral replication and neutrophils can also extrude their DNA to capture and kill bacteria and fungi, a process called neutrophil extracellular traps (NETs). In fact, an excessive NET formation and a break in NET destruction are associated with autoimmune diseases, such as SLE[63–65]. Altogether, these complex innate processes facilitate pathogens clearance, although some pathogens manage to escape elimination thus adaptive responses are extremely necessary. In fact, after a pathogen invasion, APCs have a relevant role in adaptive immunity activation, since they increase the expression of MHC and co-stimulatory molecules and migrate to lymph nodes, where can induce the differentiation of T lymphocytes, initiating adaptive immune responses[57].

In contrast, the adaptive immunity (or acquired immunity) is a late antigen-specific response that allows the creation of immunological memory, where both B and T lymphocytes are the key players[46]. Additionally, depending on the type and function of lymphocytes the adaptive immunity is subdivided into humoral immunity and cell-mediated immunity. The humoral immunity is mediated by B cells which secret antibodies that recognize microbial antigens targeting them for elimination by phagocytes and complement system. Whereas, T lymphocytes are implicated in cell-mediated immunity being subdivided in helper T lymphocytes, cytotoxic T lymphocytes and regulatory T lymphocytes, which, respectively, activates the phagocytes to destroy the pathogens, directly kills the infected cells or tumour cells and suppress immune responses. Together, B and T cells have a crucial role in the maintenance of homeostasis, although a slight alteration in immune cells function could be involved in the development and exacerbation of autoimmune diseases, such as SLE[66,67].

2.2 T lymphocytes in the etiopathogenesis of SLE – a focus on γδT cells

T lymphocytes are thymus-derived cells, that are mainly classified into two types, the αβT and γδT cells, depending on the type of T cell receptor (TCR)/CD3 complex that they express at the cell surface, the αβTCR and γδTCR, respectively (Figure 5)[46]. The αβT cells comprise the $CD8^+$ and $CD4^+$ T cells, which became activated after antigen binding presented by MHC-I and MHC-II molecules, respectively. Moreover, the $CD8^+$ T cells, also known as cytotoxic T lymphocytes, are effective in the recognition and destruction of infected/damaged cells, as well as tumour cells. $CD4^+$ T cells comprise different subpopulations: the regulatory T cells (Treg) which control immune responses; and the helper T cells (Th) that are differentiated into Th1, Th2, Th9 and Th17 depending on the expressed cytokine signature[46,68].

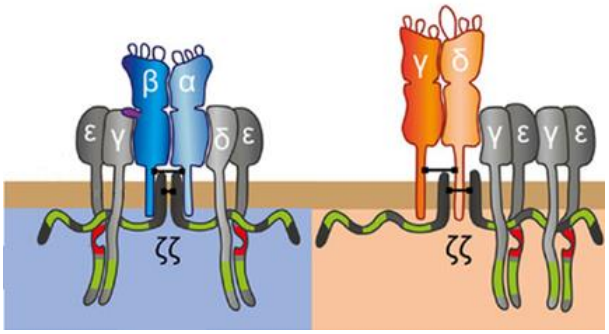

Figure 5 – The structure of human αβ and γδ T cell receptors (TCRs). The heterodimer composed by TCRα/β is identified in blue with the CD3 complex in grey, while the TCRγδ is identified in orange with the CD3 complex in grey. Both TCRs are divided into a constant (C) and a variable (V) region. Adapted from Morath *et* al., 2020[69].

The focus of this thesis is in γδT cells, that constitute around 1% to 5% of $CD3^+$ T cells in the peripheral blood of an adult human, however, in tissues, this percentage differs substantially[70]. The human γδTCR is an heterodimer composed by a γ and δ polypeptide chains associated with a CD3 complex of transmembrane proteins (Figure 5). The human TCRγ and TCRδ genes comprise a constant (C), joining (J) and variable (V) gene segments, in which the TCRδ gene has an additional diversity (D) gene segment that provide diversity to the γδTCR[71,72]. The human TCRδ chain display three variable regions, the Vδ1, Vδ2 and Vδ3, while the human TCRγ chain exhibit at least nine variable regions (Vγ), as so, γδT cells are classified accordingly to the variable regions that they express[71,73]. Moreover, each subset displays different tissue distribution (Table 1).

Table 1 – Peripheral distribution of human γδT cell subsets. Adapted from Bonneville *et* al., 2010[74].

Human subset	Peripheral tissue distribution
Vδ1	Thymus, spleen, liver, gut epithelium, and dermis
Vδ2 (mainly Vγ9Vδ2)	Peripheral blood
Vδ3	Liver and gut epithelium

The γδT cells play an important role in the immune system and are frequently called "the bridge" between innate and adaptive immunity. On one hand, they are involved in the first line of defence with a rapid innate-like response against pathogens, as well as acting as antigen-presenting cells. In fact, these cells can recognize and present antigens and display costimulatory signals that are strong enough to induce $CD4^+$ and $CD8^+$T cell proliferation and differentiation[75]. On the other hand, these cells may also participate in the adaptive immune system by a TCR antigen-specific response and by providing help for B cells differentiation and antibodies production[76]. Additionally, these cells can recognize a variety of ligands, such as stress-induced molecules, MHC-like and non-MHC restricted molecules, soluble proteins, and small peptides[77,78]. Interestingly, γδT cells earned attention when was found that the Vγ9Vδ2 subset recognizes self and non-self antigens that no other immune cell can, which are known as phosphoantigens - small non-peptide phospho-containing molecules involved in the metabolic pathway of microbial and eukaryotic organisms[79,80]. Therefore, the γδT cells and other T cell subsets are so involved in the immune system, that any change in cellular and molecular mechanisms translate into an altered cell phenotypes and abnormalities in the production of cytokines, which may favour SLE and other autoimmune diseases[81,82].

As mentioned previously, SLE is a challenging and heterogeneous disease in which the exact molecular and cellular mechanisms that underlie this disease remain unknown. However, there is some evidence of how the dysregulation of immune responses and the breakdown of self-tolerance are factors that contribute to the development of SLE. In the immunopathogenesis of SLE has been observed an accumulation of apoptotic material containing nucleic acids due to defects on apoptotic cell clearance[83]. In addition, some subsets of T cells, such as γδT cells are accumulated in the kidney from SLE patients and reduced in peripheral blood from these patients, suggesting a role of γδT cells in the pathogenesis of SLE[84,85]. Interestingly, the cytokine IL-17, one of the most studied cytokines, induces pro-inflammatory effects and is mainly produced by $CD4^+$Th (Th17)[86]. Although, in inflammatory responses, innate immune cells are the major sources for this cytokine, where the γδT cells are one of the most crucial ones[87,88]. Moreover, in serum and blood from SLE patients, was observed increased amounts of

IL-17, IL-10, interferon(IFN)-gamma (IFNγ) and type-I IFN[89,90]. Additionally, patients also displayed an increased proportion of Th17 and low amounts of IL-2 a cytokine responsible for Treg:Th17 cell balance[91–93]. As so, authors associate the low IL-2 amounts with enhanced generation of IL-17-producing T cells, which creates a pro-inflammatory environment and instigate SLE pathogenesis[94]. Moreover, other studies demonstrated an aberrant T cell signalling in SLE cells, resulting in reduced IL-2 levels and TCR hyperactivation[95–97]. Alongside with the pathogenic role of T cells, the γδT cells are also involved in inflammation and autoimmunity, such as autoimmune rheumatic diseases[98,99]. In SLE, γδT cells display an abnormal expression, as well as an aberrant cytokine profile. Studies showed increased expression of intracellular IFNγ, IL-4, IL-10, and transforming growth factor-β in peripheral SLE γδT cells, as well as an enhanced percentage of γδT cells that express the mentioned cytokines[100]. Furthermore, T lymphocytes not only play a direct role in the immunopathogenesis of SLE but can also participate indirectly by instigating other immune cells responses. For example, was demonstrated that a subset of healthy γδT cells that express the chemokine receptor CXCR5, provide B cell help for antibodies production, suggesting a role in the interplay between γδT and B cells in SLE pathogenesis[101]. Moreover, as already mentioned, was observed increased levels of IFNγ in T and γδT cells from SLE patients, and the authors described that the high amounts of IFNγ observed induced the production of BAFF and the hyperactivation of B cells[102]. Consequently, in SLE, the differentiated B cells can secrete autoantibodies against autoantigens and instigate inflammatory responses against own body, as well as the recruitment of inflammatory cells, such as NK cells, macrophages and more T cells[103]. Following this, immune complexes can be deposited in tissues and contribute to tissue and organ damage, one of the major problems in SLE[24].

In summary, γδT cells are an important subset of immune cells, which the role in SLE immunopathogenesis remains to be clarified. Therefore, it is of utmost importance the identification and characterization of the mechanisms underlying the impact of γδT cells in the immunopathogenesis of SLE.

3. Protein Glycosylation

The field of Glycobiology has been growing since the early 20th century, involving the study of the structure, biosynthesis and biological functions of saccharides or glycans[104]. Most cells are covered with a dense coat of glycans (glycocalyx) and the repertoire of glycans and their derivates represent the organisms' glycome. Nowadays, it is predicted that the glycome presents much more complexity than the genome or proteome, revealing the importance of glycans in Health and Life Sciences[104,105]. The term glycomics has emerged to investigate, analyse and fulfil some tools that enable the study of glycans and glycan-binding proteins[106–108]. Glycans contribute to a variety of important biological functions, influencing cell-cell and cell-matrix interactions, as well as protein function, signalling and trafficking[109]. Additionally, are crucial components of the immune system, allowing the correct recognition of cells and pathogens by the immune cells.

Glycosylation is a ubiquitous post-translational modification in which glycosidic linkages of carbohydrates (glycans) are formed to other saccharides, proteins, or lipids through the action of diverse enzymes, resulting in different families of glycoconjugates (Figure 6)[110]. This enzymatic process occurs in the lumen of the endoplasmic reticulum (ER) and in the Golgi apparatus (GA) and it is essential for protein folding, structure and function. Furthermore is a highly regulated mechanism dependent on the expression and localization of different glycosyltransferases and glycosidases enzymes, along with substrate availability[111]. The two main types of protein glycosylation involve *N*-glycosylation and *O*-glycosylation, which comprises different types of glycans linked to a polypeptide backbone, via nitrogen (*N*-glycans) or oxygen (*O*-glycans) linkages, respectively. *N*-linked glycans are attached to asparagine (Asn) residues of proteins via nitrogen linkages, at a consensus peptide sequence Asn-X-Serine(Ser)/Threonine(Thr), where X is any amino acid excepting a proline, while *O*-linked glycans are attached to the oxygen atom of a Ser or Thr residues and are particularly found in mucins[104].

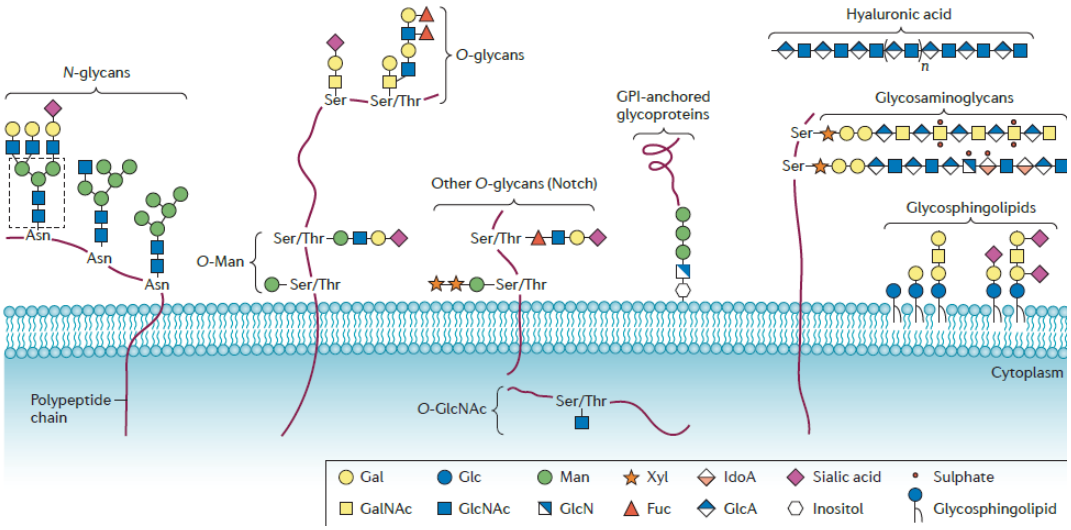

Figure 6 – Glycoconjugates in mammalian cells. Glycans are attached to other saccharides, proteins or lipids forming different classes of glycoconjugates, such as *N*-glycans, *O*-glycans, glycosaminoglycans, glycosphingolipids and glycosylphosphatidylinositol (GPI)-anchored glycoproteins. Adapted from Pinho *et* al., 2015[110].

3.1 *N*-glycosylation

N-glycans share the same core structure mannose$(Man)_3$*N*-Acetylglucosamine$(GlcNAc)_2$Asn-X-Ser/Thr and can be modified into three major final structures: high-mannose or oligomannose *N*-glycans, which only Man residues extend the core; complex *N*-glycans, that can have up to six branches initiated by GlcNAc residues; and hybrid *N*-glycans, in which Man residues elongate the core in α1-6Man arm and one or two GlcNAcs are attached to the α1-3Man arm (Figure 7)[104].

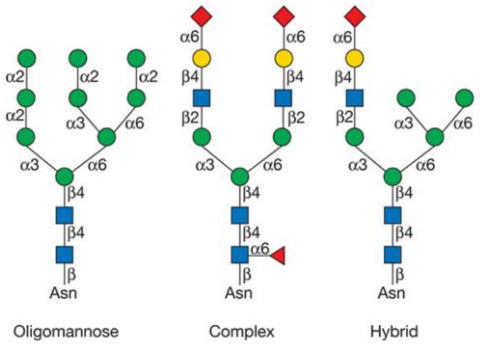

Figure 7 – Types of *N*-glycans. All *N*-glycans share the same core structure $Man_3GlcNAc_2$ Asn-X-Ser/Thr, and are classified as oligomannose or high-mannose *N*-glycans, complex *N*-glycans and hybrid *N*-glycans[104].

The first step of *N*-glycan biosynthesis occurs in the ER membrane of all eukaryotic cells, where one GlcNAc residue is transferred to a lipid carrier, known as dolichol phosphate (Dol-P) (Figure 8)[104]. Then, a second GlcNAc along with five Man residues are attached to the glycan. This oligosaccharide is translocated to the cytoplasmic side of the ER membrane and is extended by the addition of more four Man and three glucose (Glc) residues, resulting in an *N*-glycan precursor. Lastly, this precursor is transferred by

oligosaccharyltransferase (OST) to Asn residue in the consensus Asn-X-Ser/Thr sequence of proteins that have translocated across the ER membrane. In the lumen of the ER, the glycoprotein is trimmed by glucosidases and mannosidases which, respectively, remove three Glc residues and one Man residue, allowing the correct protein folding. Finally, glycans are submitted to a quality control mechanism assisted by two lectins – calnexin and calreticulin – where, if the glycoprotein is correctly folded continues to the GA or if it is misfolded are targeted for degradation by ER-associated degradation (ERAD) pathway[112].

Once in the GA, two glycosidases, α-1,2 mannosidase IA and IB (α-Man IA and IB) encoded by mannosidase alpha class 1A member 1 and 2 (*MAN1A1* and *MAN1A2* genes), trims Man residues producing an intermediate of complex and hybrid *N*-glycans – the $Man_5GlcNac_2$ (Figure 8)[104]. From here on, this glycan isomer is elongated through the action *N*-acetylglucosaminyltransferase I (GnT-I), encoded by mannosidase acetylglucosaminyltransferase 1 (*MGAT1*), which is the first glycosyltransferases that initiate *N*-linked carbohydrate formation by the addiction of GlcNac residues. Further, α-mannosidase II enzymes (α-Man IIA and IIB) encoded by mannosidase alpha class 2A member 1 and 2 (*MAN2A1* and *MAN2A2*) removes two-terminal mannose residues (α1-3Man and α1-6Man) and GnT-II (encoded by *MGAT2*) add a GlcNAc residue to the α1-6Man, resulting in a precursor of complex *N*-glycans. Hybrid *N*-glycans are formed if α-Man II does not act in the $GlcNacMan_5GlcNac_2$ glycan, resulting in $GlcNAcMan_4GlcNAc_2$ hybrids. While, complex *N*-glycan branching is initiated by the action of GnT-IV (encoded by *MGAT4*) and/or GnT-V (encoded by *MGAT5*), where is added a β1,4GlcNAc branch to the α1-3Man arm and/or a β1,6GlcNAc to the α1-6Man arm, respectively, resulting in tri- and tetra-antennary *N*-glycans. Additionally, hybrid and complex *N*-glycans may also carry a bisecting GlcNAc residue linked to the β-Man by the action of GnT-III (encoded by *MGAT3*). However, the presence of this bisecting GlcNAc inhibits the elongation[113]. Finally, glycans are maturated in the trans-Golgi where hybrid and complex *N*-glycans can be elongated and decorated by additional sugars, for example, galactose, fucose, *N*-acetylgalactosamine, poly-*N*-acetyllactosamine (poly-LacNAc) and sialic acid, providing a huge and diverse panel of sugars[104].

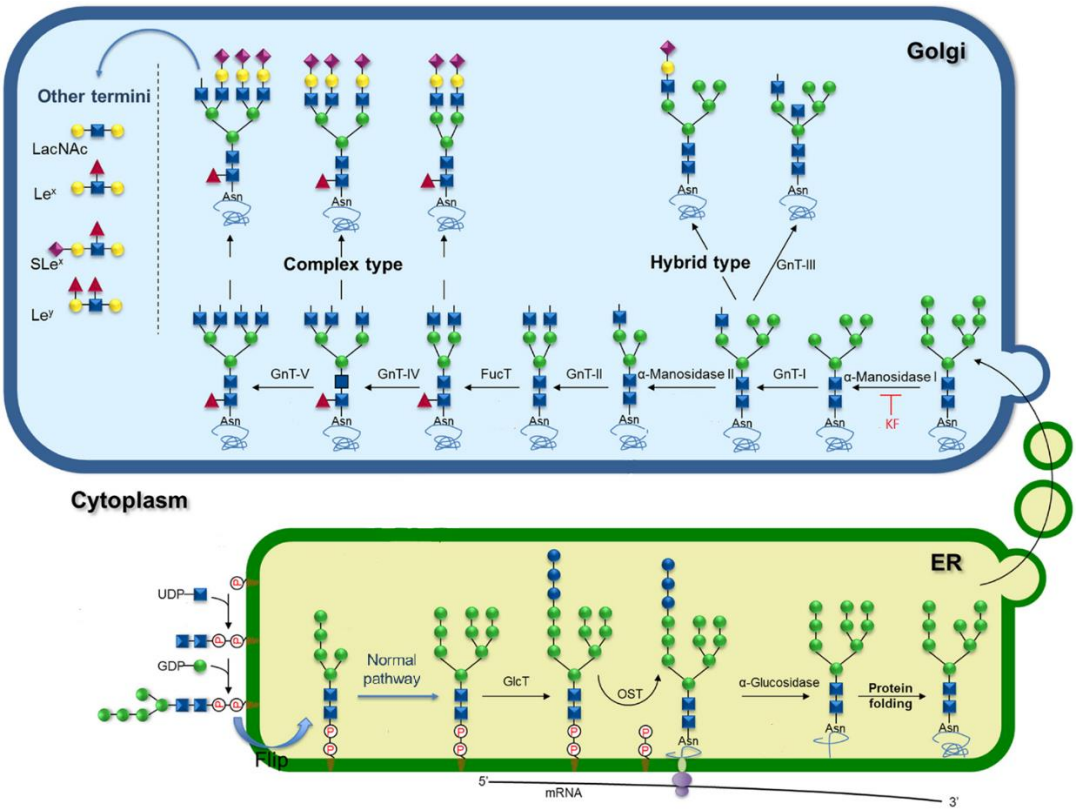

Figure 8 – *N*-glycosylation pathway in Endoplasmic Reticulum (ER) and Golgi Apparatus (GA) secretory pathway. The *N*-glycans synthesis is initiated in the ER, where a *N*-glycan precursor is transferred to Asn residue in the consensus Asn-X-Ser/Thr sequence by oligosaccharyltransferase (OST). Then, occurs a sequential remove of glucose residues by α-glucosidases and one Man residue by ER α-mannosidase. The glycoprotein is submitted to a quality control mechanism assisted by calnexin and calreticulin and if it is correctly folded moves to the GA. There, is trimmed by α-1,2 mannosidases IA and IB to remove the Man residues, producing the intermediate of complex and hybrid *N*-glycans. Then, GnT-I initiate the addiction of the first GlcNAc residue and α-mannosidase II enzymes removes terminal Man residues. Hybrid *N*-glycans are formed when α-mannosidases II does not act. Further GnT-I action, a complex *N*-glycan precursor is formed through the addition of a GlcNAc residue by GnT-II. Finally, by the action of GnT-IV and GnT-V the complex *N*-glycans are formed. Both complex and hybrid *N*-glycans can be elongated and decorated by additional sugars, such as galactose and sialic acid. It is also evidenced an α-mannosidase I inhibitor, kifunensine (KF). Adapted from Reily *et* al., 2019[111].

3.2 Role of glycans in immune responses

Glycans are well-known to be implicated in immune modulation since almost all immune receptors are glycosylated, including PRRs, toll-like receptors, proteins from MHC complex, T and B cells receptors and co-receptors, as well as, chemokines and cytokine receptors[114]. Importantly, glycosylation demonstrates a crucial role in host defence by interacting with glycan-binding proteins and it is also implicated in T cell biology, for example, in T cell development, differentiation and signalling[115]. As briefly mentioned, T lymphocytes receptors are highly glycosylated and for proper immune activation, they rely on co-stimulatory and co-inhibitory signals, as well as on MHC complex to present the majority of antigens[116]. Some evidence shows the importance of glycosylation for the correct antigen-recognition and antigen-presentation and their

influence on immune cell response. Ovalbumin (OVA) is an antigen model which is used to trigger an immune response. Changes in OVA glycosylation (with Lewis^X glycans) lead to the priming of CD8$^+$T cell proliferation, as well as, Th1 differentiation[117]. In addition, a conditional *Mgat2* knock-out mice, with decreased expression of branching *N*-glycans on MHC-II complex showed decreased antigen binding and presentation, influencing the T cell activation[118]. CD28 is a co-stimulatory receptor and a glycoprotein expressed by T cells and necessary for T cell proliferation through the binding to CD80/CD86 molecules present in APCs. Interestingly, some studies described that point mutations of *N*-glycosylation sites in CD28 (resulting in reduced CD28 glycosylation), led to a higher affinity of CD28 to CD80, as well as induced IL-2 signalling, highlighting the role of glycans in TCR response[119]. The cytotoxic T-lymphocyte protein 4 (CTLA-4) is an important co-inhibitory receptor, modulated by *N*-glycosylation, that is associated with suppression of T cell activity and immune homeostasis[120,121]. After TCR activation, the recovery of immune tolerance is achieved by an increase of β1,6-GlcNAc branching in CTLA-4 associated with cell surface retention of the receptor, and consequently, the inhibition of T cell activity[121]. However, the opposite is observed when β1,6-GlcNAc branching is reduced on T cells, where intracellular CTLA-4 increases and surface retention decreases, leading to T cell hyperactivation[122]. *N*-glycosylation encompasses a huge impact in the immune system by modulating TCR activation threshold. Researchers found in mice depleted for *Mgat5* (lacking β1,6-GlcNAc branched *N*-glycans) an enhancement on T cell activation by an increased TCR clustering and a lower T cell activation threshold[123]. This is also supported by the absence of “lattice” structures (LacNAc) between the TCR and galectin-3 (a carbohydrate recognition protein), which in homeostatic conditions should prevent the TCR clustering[123,124].

Other components of the immune system are the glycan-binding proteins (GBPs)[104]. The interaction between GBPs and glycans is essential for the recognition of pathogens by the immune system, as well as for antigen presentation and T cell modulation[109,114]. GBPs are characterized by the expression of one or more carbohydrate-recognition domains and include C-type lectins, siglecs and galectins[104]. The C-type lectins (CTLs) are soluble or membrane-bound proteins that bind to carbohydrates in a Ca^{2+}-dependent manner, although some findings indicate a calcium-independent binding to other ligands[125]. CTLs are divided into 17 subgroups based on phylogeny and structure, where the main ones are (Figure 9): DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN); macrophage inducible C-type lectin (Mincle); dendritic cell-associated C-type lectin 1 and 2 (Dectin-1 and -2); macrophage mannose receptor (also known as mannose receptor, MR); mannose-binding protein (MBL); and macrophage

galactose lectin (MGL)[126,127]. Moreover, CTLs binds to carbohydrates through conserved amino acid motifs. The EPN (Glu-Pro-Asp) motif is characteristic of mannose- and/or fucose-binding and includes DC-SIGN, MR and Mincle[128]. In contrast, the QPD (Gln-Pro-Asp) motif promotes binding-affinity for galactose- or *N*-acetylgalactosamine-type carbohydrates and includes the MGL[129,130]. Additionally, the interplay between glycans and CTLs has a major role in immune responses, through the regulation of host defence, immune tolerance, inflammatory responses and autoimmunity[109]. DC-SIGN, also known as CD209, is a CTL expressed mainly in macrophages and DCs that recognize glycoproteins with high-mannose and fucose residues[131]. DC-SIGN is involved in presentation of host antigens through MHC complex to T cells, enhancing immune responses[132]. In fact, it was observed in mouse model that after a host infection by pathogens expressing mannose (like *Mycobacterium tuberculosis*), DC-SIGN activates Raf-1 pathway, leading to the release of pro-inflammatory cytokines[133]. The same was observed for HIV-1, where DC-SIGN is a receptor that binds to HIV-1 envelope glycoprotein, also promoting the release of pro-inflammatory cytokines[134]. This receptor has been also involved in some autoimmune and inflammatory diseases by showing a protecting function in type 1 diabetes or enhancing the pathogenesis of allergys[135,136]. MR is another CTL that contributes to a Th17 response in fungal infections, although, in allergies favours a Th2 response[137,138]. Furthermore, MR seems to show a protective role in mice with rheumatoid-like disease[139].

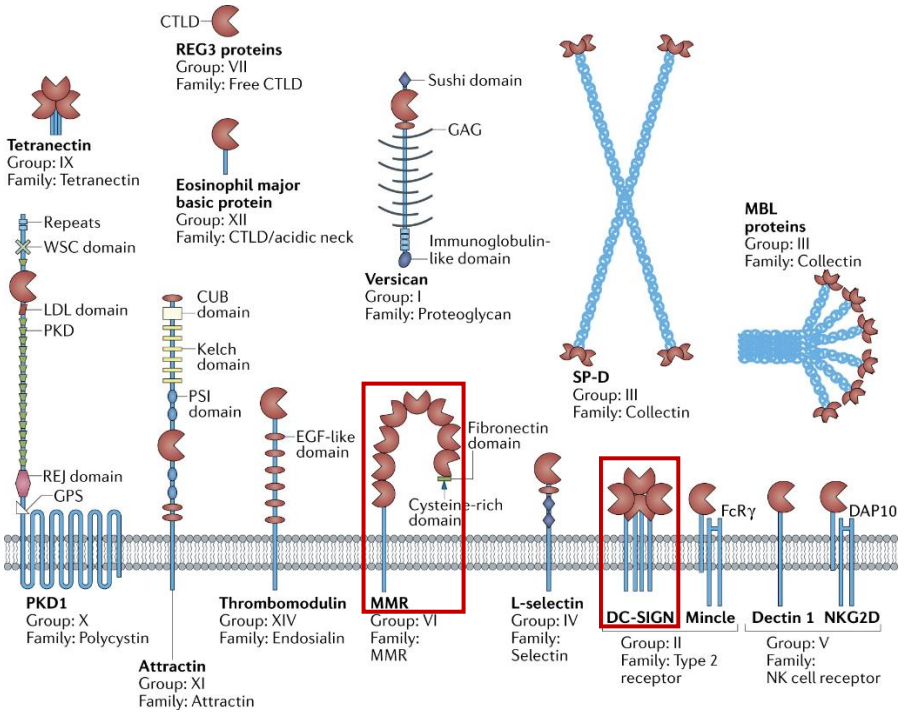

Figure 9 – Principal types of soluble and membrane-bound C-type lectins (CTLs). Highlighted are macrophage mannose receptor (MR, MMR in figure) and DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), which will further be considered in this work. Adapted from Brown *et* al., 2018[127].

3.3 Glycoimmunology in autoimmune diseases

The dysregulation of the glycosylation pathway has been associated with development of some autoimmune diseases, like RA, inflammatory bowel disease (IBD) and multiple sclerosis (MS). However, the role of protein glycosylation in SLE pathogenesis is far from being elucidated. Mice deficient on α-Man II revealed a lupus-like glomerular nephritis phenotype associated with a deficit in complex *N*-glycans[140]. Similarly, it was observed in *MRL-lpr* mouse model (spontaneously develop SLE-like phenotype) a lower abundance of complex *N*-glycans[141]. Additionally, the *Mgat5*-null mice showed an increased susceptibility to experimental autoimmune encephalomyelitis (EAE) and to ulcerative colitis (UC), evidencing the impact of glycosylation's alterations in autoimmunity[116,124,144]. Interestingly, after GlcNAc supplementation, both disease susceptibility and severity decreased, demonstrating once again, the immune-modulatory capacity of glycans[143,144]. Moreover, our group demonstrated a diminished *MGAT5* gene expression (deficit in β1,6 branched *N*-glycans) in T lymphocytes from UC patients and a correlation with TCR hyperactivity and disease severity[145,146]. The *ex vivo* supplementation with GlcNAc of T cells from UC patients showed a controlled T cell activity with increased branched *N*-glycans and a decreased TCR activation, proposing a target-specific glycan therapy[144]. Furthermore, immunoglobin G (IgG) is secreted by plasma cells and is also glycosylated[147]. Interestingly, studies in SLE, IBD and RA

patients revealed an abnormal galactosylation of the IgG, with a higher abundance of agalactosylated IgG glycans, which was also correlated with disease activity[148–150]. More recently, our group showed that *MGAT5* polymorphisms seem to be associated with the changes in T cells and IgG glycosylation in UC patients[146]. *MGAT5* polymorphisms were also documented in MS[151]. Taken together, changes in glycosylation has been demonstrated to impact directly or indirectly in both innate and adaptive immune responses, associated with the development and progression of autoimmune diseases.

Despite relevant in an autoimmune context, poor is known regarding the impact of glycosylation modifications in the activity and function of γδT cells and its consequences in SLE pathogenesis. As already mentioned, γδT cells express a γδTCR that is associated with a CD3 complex which comprises the CD3ε, CD3γ, CD3δ and CD3ζ. Interestingly, the CD3δ from γδTCR contains complex *N*-glycans and the CD3γ display lower sialylation, when compared with αβTCR-CD3 complex[152]. Evidences showed that the deglycosylation of the γδTCR improve the CD3 conformational change that occurs after antigen binding, indicating that deglycosylation might enhance γδTCR clustering[153]. Moreover, the γδTCR is dependent on costimulatory molecules for γδT cells activation, such as CD28[154,155]. Interestingly, when glycosylation of CD28 is diminished, the CD28-CD80 binding increases, suggesting a role of glycosylation alterations in co-stimulatory receptors that may lead to γδT cell hyperactivation[119]. Additionally, in the kidney of SLE patients it was observed a hyperactivated and accumulation of these cells[85]. Another study showed that activated SLE T cells bind significantly less to galectin-1 (an immunoregulatory lectin) due to sialylation on T cells surface[156]. Altogether, these evidences suggest an important role of glycosylation on γδT cells, highlighting the importance in understanding the mechanisms underlying the impact of *N*-glycosylation alterations in γδT cell function and response, as well as its association with autoimmunity, such as SLE.

Aim

The goal of this work is to unravel how changes in cellular glycosylation modulate and define γδT cells activity and function, exploring its impact in SLE immunopathogenesis. For that, we propose five specific objectives:

i. To evaluate the expression levels of γδT cells *in situ*, in clinical samples from SLE patients and in samples from an autoimmune disease-susceptible mouse model;
ii. To characterize the expression levels and the glycosylation profile of γδT cells in the periphery of SLE patients;
iii. To evaluate *in vivo* the glycosylation pattern of γδT cells in different environments and to assess the role of γδT cells in the development/exacerbation of autoimmune-like syndrome in *Mgat5*$^{-/-}$ mouse model;
iv. To re-program γδT cells glycosylation assessing the impact in the modulation of immune response;
v. To evaluate the expression of glycan-binding receptors at the surface of γδT cells in homeostasis and disease.

Material and methods

1. Patients' Cohort and Human Samples

The formalin-fixed paraffin-embedded (FFPE) kidney samples from SLE patients diagnosed with lupus nephritis were obtained from the Nephrology Department of Porto Centre Hospital-Hospital Santo Antonio (CHP/HSA), Porto, Portugal, and Nephrology Department of Coimbra University Hospital (CHUC), Coimbra, Portugal. For the controls we used kidney sections from healthy donors (excluding autoimmune, cancer and inflammatory-mediated diseases) obtained from the Pathology Department of São João University Hospital (CHSJ), Porto, Portugal.

The peripheral blood from SLE patients and healthy volunteers were obtained from Clinical Immunology Department of CHP/HSA, Porto, Portugal. The buffy coats from healthy donors, used for γδT cells isolation an *in vitro* reprogramming, were obtained from Blood Bank of CHSJ, Porto, Portugal. All the collected samples were ethically approved, and all participants gave informed consent.

2. Human PBMCs and γδT cells isolation

The peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of SLE patients and healthy controls, as well as healthy donors' buffy coats by a density gradient centrifugation using Lymphoprep™. The 2 volume of blood was carefully added on top of 1 volume of Lymphoprep™. Then, the samples were centrifuged for 20 min at 900 *g* without brake or acceleration, at room temperature (RT). Finally, the PBMCs interface was aspirated and cells were washed with Phosphate Buffer Saline (PBS, pH=7.4) and centrifuged for 10 min at 300 *g* and 4 ºC. When necessary, the samples were incubated with ammonium chloride potassium (ACK) erythrocyte lysing buffer (from in-house made 10x buffer: 1.5 M ammoniumchlorid (NH_4Cl), 100 mM kaliumhydrogencarbonat ($KHCO_3$), 10 mM EDTA; all from Sigma-Aldrich), for 3 min. Then, cells were centrifuged for 10 min at 300 *g* and 4 ºC and washed with PBS and centrifuged again, as previously. The same was performed in peripheral blood from healthy buffy coats, with the exception that initially blood was diluted 1:4 with PBS.

Then, the $CD3^+$T cells from PBMCs of healthy donors' buffy coats were magnetically sorted using the EasySep™ Human T Cell Enrichment Kit (STEMCELL Technologies), following the manufacturer's instructions. Subsequently, the γδT cells

were also magnetically sorted the by EasySep™ Human Gamma/Delta T Cell Isolation Kit (STEMCELL Technologies), according with manufacturer's instructions.

3. Human γδT cells culture

The sorted γδT cells were cultured for 24 h, at 37 ºC in 96-well round bottom plates with 200 µL of *Roswell Park Memorial Institute* (RPMI) medium supplemented with 10% fetal bovine serum (FBS), 1% Penicillin-streptomycin (Pen-Strep) and plate-bound anti-CD3 monoclonal antibody (mAb) (clone OKT3) (0.5 µg/mL), as well as soluble anti-CD28 mAb (clone CD28.2) (0.5 µg/mL) (eBioscience™). Then, for the ablation of complex *N*-glycans, it was added kifunensine (KF; 20 µM) (Sigma-Aldrich) to the γδT cells' cultures, a α-mannosidase I inhibitor. At the selected timepoint, the supernatants were collected and snap-frozen and the cells were stained for flow cytometry.

4. Animal experiments and sample preparation

Animal models

All mouse procedures were approved by i3S ethics committee for animal experimentation under Portuguese regulations. The present study includes the following animal models - C57BL/6J wild-type mice (*Mgat5*-WT, The Jackson Laboratory) and C57BL/6J *Mgat5*$^{-/-}$ mice (*Mgat5*-knock-out (KO), kindly provided by Michael Pierce, University of Georgia, Athens, GA), as well as C57BL/6J *Rag1*$^{+/+}$*Mgat2*$^{fl/f}$ mice (*Mgat2*-WT, kindly provided by Michael Demetriou, University of California, Irvine, CA) and C57BL/6J *Rag1*$^{Cre/+}$*Mgat2*$^{fl/f}$ mice (*Mgat2*-cKO, kindly provided by Marc Veldhoen, IMM, Lisbon, Portugal) a mouse model lacking the *Mgat2* gene in recombination activating 1 (*Rag1)*-expressing cells – that were housed at i3S animal facility.

Sample preparation

The *Mgat5*-WT and -KO mice with 8 weeks-old (new) and 15 months-old (aging) and *Mgat2*-WT and -cKO mice with 8 weeks-old were sacrificed in a CO_2 chamber. In *Mgat5* (new and aging) mice the colon, kidney and lung were aseptically removed, with the addition of spleen for *Mgat5*-new mice and mesenteric lymph nodes (MLNs) for *Mgat5*-aging mice. In *Mgat2*-WT and -cKO mice only the spleen was collected and added to Hanks' Balanced Salt Solution (HBSS) modified medium (without calcium chloride and magnesium sulfate; Sigma-Aldrich), for posterior processing. In *Mgat5* (new and aging) mice, the colon and kidney were bisected longitudinally in equal portions (portion A and B). One portion (portion A) and the remaining organs were transferred to 6-well plate with HBSS modified medium, for posterior processing. The other portion (portion B) from *Mgat5*-new mice was fixed in 4% formaldehyde (PanReac ApplieChem) for FFPE

samples processing by histology service at i3S. The portion B from kidney of *Mgat5*-aging mice was cut transversely and one half was culture for 18 h with RPMI medium (without FBS) to further cytokine analysis and the other half was incubated in 4% formaldehyde for paraffin inclusion. From the portion B of the *Mgat5*-aging' colon, the distal part was removed and cultured with RPMI medium (without FBS) and the remaining part was fixed and processed, both as described previously.

5. Isolation of the mice mononuclear and γδT cells

For lymphocytes isolation, the portion A from the colon and kidney and the lung were cut into small pieces and incubated with digestion solution, including RMPI medium supplemented with 0.5 M $CaCl_2$, 0.5 M $MgCl_2$ and 1 mg/mL Collagenase IV (Sigma-Aldrich), for 60 min and under agitation at 37 ºC. After 60 min, was added 1 volume of RPMI medium with 10% FBS and 2 mM EDTA, to stop the digestion. The spleen of both animal models (*Mgat5* and *Mgat2* mice) and the MLNs from *Mgat5*-aging mice were smashed into two frosted microscope slides and was added PBS. All mentioned samples were passed through a 70 µm cell strainers, with the help of a syringe plunger, centrifuged for 10 min at 300 *g* and 4 ºC and the pellet was resuspended in PBS. The cell suspension from colon, kidney and lung was carefully added on top of Lymphoprep™ solution in 2:1 ratio and centrifuged for 20 min at 900 *g* without brake or acceleration, at RT. Finally, the mononuclear cell interface was aspirated, and cells were washed with PBS and centrifuged for 10 min at 300 *g* and 4 ºC. When necessary, the samples were incubated with ACK lysing buffer at RT. After 3 min, cells were centrifuged for 10 min at 300 *g* and 4 ºC, washed with PBS and centrifuged as previously. The cells were then ready for flow cytometry staining.

From the splenocytes of *Mgat2* mice, the $CD3^+$T cells were magnetically sorted using the EasySep™ Mouse T Cell Isolation Kit (STEMCELL Technologies), following the manufacturer's instructions. Then, the γδT cells were isolated through the flow cytometry sorting (BD fluorescent-activated cell sorting (FACS) Aria II, BD Biosciences, US), selecting negative fixable viability dye (FVD) and $\gamma\delta TCR^+$ $\alpha\beta TCR^-$ (Table 2).

6. Mice γδT cells culture

The sorted γδT cells from *Mgat2* mice splenocytes (WT and cKO) were cultured in 96-well round bottom plates with 200 µL of RPMI medium (with 10% FBS and 1% Pen-Strep), soluble anti-CD28 mAb (clone CD28.2) (0.5 µg/mL) (eBioscience™) and plate-bound anti-CD3 mAb (clone 145-2C11) (0.5 µg/mL) (eBioscience™), for 24 h, at 37 ºC. At the selected timepoint, the cells were stained for flow cytometry.

7. Enzyme-Linked Immunosorbent Assay (ELISA)

The supernatants of renal explants from *Mgat5* aging mice were collected and IL-17A and IFNγ (Invitrogen, CA, USA) cytokines were analysed by ELISA assay, following the manufacturer's instructions. The plate was measured at 450 nm and 570 nm using the Biotek MQX200 uQuant Microplate Reader. The cytokine concentration was expressed as pg/mL/mg of tissue.

8. Flow Cytometry

PBMCs of SLE patients, mononuclear cells from *Mgat5*$^{-/-}$ mouse model (young and aging), sorted γδT cells from healthy donors' buffy coats or from *Mgat2*$^{-/-}$ mice, as well as the respective controls were washed with PBS, centrifuged at 300 *g* for 5 min at 4 ºC and stained with FVD for 30 min on ice and dark. All the used antibodies and lectins are mentioned in Table 2. Following, cells were washed with PBS and stained with fluorescent or biotinylated lectins for 15 min on ice and dark. The selected lectins includes: *Phaseolus vulgaris Leucoagglutinin* lectin (L-PHA), that specifically recognizes β1,6-GlcNAc branched *N*-glycans; *Galanthus nivalis* lectin (GNA), that specifically recognizes terminal α-1,3-linked mannose; Lycopersicon Esculentum lectin (LEL), that recognizes polylactosamine structures; and Sambucus Nigra lectin (SNA), that specifically recognizes the α-2,6-linked sialic acid. After 15 min, cells were washed with FACS buffer (PBS with 1% FBS) and mice cells were blocked with 2% normal rat serum (STEMCELL Technologies) and human cells with 2% normal mouse serum (Jackson Immunoresearch), for 10 min on ice and dark. Following, cells were stained with a panel of surface fluorescent antibodies for 30 min, on ice and dark and then washed with FACS buffer. When necessary, cells were stained with streptavidin or/and secondary antibody (secondary AB), for 30 min on ice and dark. Finally, cells were fixed with 2% formaldehyde diluted in PBS, on ice and dark. After 30 min, cells were centrifuged, washed, resuspended in FACS buffer and analysed by FACS.

In the wells designated for intracellular cytokine analysis, the PBMCs from SLE patients, cells from all organs of *Mgat5*-new mice and from MLNs of *Mgat5*-aging mice, as well as the respective controls were initially incubated with 200 µL RPMI medium (supplemented with 10% FBS and 1% Pen/Strep) with Brefeldin A (Bref; 10 ng/mL, Sigma-Aldrich), Phorbol 12-myristate 13-acetate (PMA; 20 ng/mL, Sigma-Aldrich) and Ionomycin (Ion; 200 ng/mL Sigma-Aldrich), for 3 h at 37 ºC. As described previously, cells were further stained with lectins and extracellular antibodies and fixed. Then, these cells as well as γδT cells from healthy donors' buffy coats or from *Mgat2* mice were permeabilized with 0.5% saponin (Sigma-Aldrich), for 10 min at RT. Then, cells were

stained with intracellular antibodies diluted in permeabilization buffer (PB, FACS buffer with 0.5% saponin) and also washed with PB (Table 2, "Intracellular"). Lastly, cells were resuspended in FACS buffer for FACS analysis. All the samples were acquired on a FACSCanto II machine (BD Biosciences, US). The data files were analysed with FlowJo software (version 10.0) and the gating strategy to assess γδT cells is demonstrated in Supplementary figure 1. The results were represented in the GraphPad Prism 7 Software (GraphPad Software) and expressed as the median fluorescence intensity (MFI) of each lectin/antigen expression or percentage of cells. When described, the MFI was normalized (fold-change) to the mean value of the controls of each experiment.

Table 2 – List of lectins and anti-human and anti-mouse antibodies used in this work for flow cytometry staining, as well as the respective conjugate molecule, dilution, clone and company.

Specie	Lectins/ Antibodies	Conjugate	Dilution	Clone	Company
Human	TCRγδ	PerCP-Cy5	1:100	B1.1	eBioscience
Human	TCRβ	PE	1:50	BW242/412	Miltenyi Biotec
Human	CD69	PE	1:100	FN50	Biolegend
Human	CD25	PE	1:100	BC96	Biolegend
Human	HLA-DR	PE-Cy7	1:100	L243	eBioscience
Human	CD206 (MR)	PE-Cy7	1:100	15-2	Biolegend
Human	CD209 (DC-SIGN)	-	1:100	AHP627	Bio-Rad
		Secondary AB: swine anti-rabbit IgG - FITC	1:20	E0354	Agilent Dako
Human (Intracellular)	IL-17	eFluor450	1:100	eBio64DEC17	eBioscience
Human (Intracellular)	IFNγ	APC	1:200	4S.B3	eBioscience
Human (Intracellular)	IL-10	PE-Cy7	1:200	JES3-9D7	eBioscience
Mouse	TCRγδ	eFluor450	1:400	eBioGL3	eBioscience
Mouse	TCRβ	PE	1:800	H57-597	eBioscience
Mouse	CD69	PerCP-Cy5	1:200	H1.2F3	eBioscience
Mouse	CD206 (MR)	PE-Cy7	1:100	MR6F3	eBioscience
Mouse (Intracellular)	IL-17	PE	1:100	eBio17B7	eBioscience
		FITC	1:100	TC11-8H4	Life Tech
Mouse (Intracellular)	IFN-γ	APC	1:100	XMG1.2	BD Biosciences
Mouse (Intracellular)	IL-10	PE	1:100	JES5-16E3	BD Biosciences
Mouse (Intracellular)	IL-22	PerCP-eFluor 710	1:100	1H8PWSR	eBioscience
Human/Mouse	FVD	APC-eFluor 780	1:2000	-	eBioscience
Human/Mouse	L-PHA	FITC	1:1000	-	Vector Laboratories
Human/Mouse	GNA	FITC	1:1000	-	Vector Laboratories
Human/Mouse	LEL	APC	1:1000	-	Vector Laboratories
Human/Mouse	SNA	APC	1:1000	-	Vector Laboratories
Human/Mouse	GNA	Biotinylated	1:1000	-	Vector Laboratories
Human/Mouse	LEL	Biotinylated	1:1000	-	Vector Laboratories
Human/Mouse	Streptavidin	PE	1:200	-	eBioscience
		PE-Cy7	1:200	-	Invitrogen

PerCP: peridinin chlorophyll protein; **Cy5 or 7:** Cyanine 5 or cyanine 7; **PE:** phycoerythrin; **APC:** allophycocyanin; **FITC:** fluorescein isothiocyanate; HLA-DR: MHC-II cell surface receptor.

9. Databases for *in silico* analysis

The Human Protein Atlas database (http://www.proteinatlas.org) was used for an *in silico* analysis to evaluate the transcription levels of different human protein-coding genes, namely glycogenes of γδT cells and naïve $CD4^+$T cells, as well as C-type lectins genes of γδT cells. The data correspond to RNA-seq analysis of six healthy human blood samples obtained from the - Blood Atlas, consensus dataset[157]. The values were represented in the GraphPad Prism 7 Software (GraphPad Software) and are expressed as pTPM values (transcripts per million), giving the quantification of the gene abundance in different genes and samples.

The Universal Protein Resource (UniProt) database was used for an *in silico* analysis to evaluate the number of *N*-glycosylation sites in each chain of γδTCR, collected from - Post-translational modifications section[158].

10. Immunohistochemistry

For the immunohistochemistry, FFPE kidney slides from SLE patients, as well as FFPE kidney and colon slides from *Mgat5*-aging mouse model were used for γδTCR immunohistochemistry detection with UltraVision™ Quanto Detection System HRP (horseradish peroxidase) DAB (3,3'-diaminobenzidine) (Thermo Scientific, DE, USA), according to the manufacture's protocol. The tissue sections were deparaffinized two times in xylene for 10 min and rehydrated with decreasing concentrations of ethanol solutions (twice with 100% and once with 70% ethanol) for 5 min each and followed by washing with water for 10 min. The heat-induced pre-treatment for antigen retrieval was performed in a steam cooker (Ufesa) with citrate buffer (10 mM citric acid, pH=6) for 40 min. Then, the slides were left to cool for 20 min at RT and were washed twice with PBS for 5 min each. To reduce nonspecific background staining caused by endogenous peroxidase, the slides were incubated with kit's hydrogen peroxide block for 10 min in a humid chamber and washed twice with PBS. To avoid nonspecific staining, unspecific proteins were blocked for 8 min with the kit's Ultra V Block solution for human slides and with 10% bovine serum albumin (BSA; Sigma-Aldrich) diluted in PBS for mice slides. The human primary antibody - anti-human γδTCR (clone: 5A6.E9; Invitrogen) - was diluted 1:10 in 5% Ultra V Block solution (diluted in PBS). While the mouse primary antibody - anti-mouse γδTCR (clone: eBioGL3; eBioscience) - was diluted 1:100 in 5% BSA (diluted in PBS). Both antibodies were incubated over-night, in a humid chamber at 4 ºC. Then, the manufactures protocol was followed. Lastly, counterstain was performed with hematoxylin and the slides were dehydrated (firstly in 70% ethanol followed by 100% ethanol twice and finally twice in xylol) and preserved in the appropriated mounting

medium (Entellan® new, Merck Millipore). The analysis was performed in *Fiji* software (ImageJ, US).

11. Western blot

Isolated γδT cells from healthy donnors' buffy coats treated with KF and non-treated (control) were collected and protein lysates were extracted using protein extraction buffer with 1% Triton X-100, 1% NP-40, 1% phenylmethylsulfonyl fluoride (100 mM), 1% sodium orthovanadate (Na_3VO_4; 100 mM) and 4% cOmplete tablets (25x), diluted in PBS. The Blue Protein Loading Dye with reducing agent (New England BioLabs) was added to equal amounts of protein, accordingly to the manufacturers protocol and heated at 96.5 ºC for 10 min before loading onto gel. Protein lysates were subjected to 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at 125 volts for 2 h, and finally transferred to a nitrocellulose membrane at 60 volts for 90 min. The membrane was blocked with 5% BSA diluted in PBS with 0.05% Tween20 (PBST) overnight, at 4 ºC under agitation, to prevent to prevent nonspecific binding. After washing with PBST, the membrane was incubated with biotinylated L-PHA lectin (Vector Laboratories) 1:1000 diluted in 1% BSA with PBST for 60 min at RT, to detect the β1,6-GlcNAc branched structures. For detection, the membrane was incubated with the ABC solution (Vectastain® ABC Kit, Vector Laboratories, Burlingame, USA). The target proteins were visualized by a ECL reagent (GE Healthcare, Life Sciences). Positive reaction was observed in a band with the same size as γδTCR (18 kDa, accordingly to the product information from the anti-human TCR gamma/delta mAb, 5A6.E9, Invitrogen, USA).

12. Statistics

All data were analysed using the GraphPad Prism 7 Software. In the percentage and glycosylation profile of γδT cells from SLE patients was used the non-parametric Kruskal-Wallis test with Dunn's test for multiple comparisons. In the characterization of C-type lectins in γδT cells from SLE patients, was used the t-test with Mann-Whitney test. The same t-test was performed in the characterization of activation status, C-type lectins, glycosylation and cytokines response of γδT cells sorted from healthy donors' buffy coats (KF treatment), as well as in cytokines analysis of γδT cells and ELISA from the *Mgat5* mice. In the remaining analysis of *Mgat5* mice was used the t-test with Holm-Sidak method. All the results were considered statistically significant with *p*-values of less than 0.05.

Results

1. *In situ* characterization of the expression levels of γδT cells in kidney samples from SLE patients and in samples from an autoimmune disease-susceptible mouse model

Previous observations from our group (not published) have demonstrated a decreased expression of *β*1,6-GlcNAc branched *N*-glycans in biopsy-proven lupus nephritis from SLE patients. As so, in a first approach we associated the observed alterations in the glycosylation profile of LN samples with levels of γδT cells in kidney FFPE from SLE patients. Through immunohistochemistry, we observed an increase of γδT cell inflammatory infiltrate in the kidney of SLE patients, detected by anti-human pan γδTCR mAb, when compared with kidney tissues from healthy controls (Figure 10A). Furthermore, in an autoimmune disease-susceptible mouse model, the *Mgat5*$^{-/-}$ aging mice, we observed an increase of γδT cells infiltrates in the kidney and colon samples of those mice when compared with the controls (Figure 10B). Together, these results showed an accumulation of γδT cells in kidney samples from SLE patients, as well as in kidney and colon of a glycoengineered mouse model that lack *β*1,6-GlcNAc branched *N*-glycans.

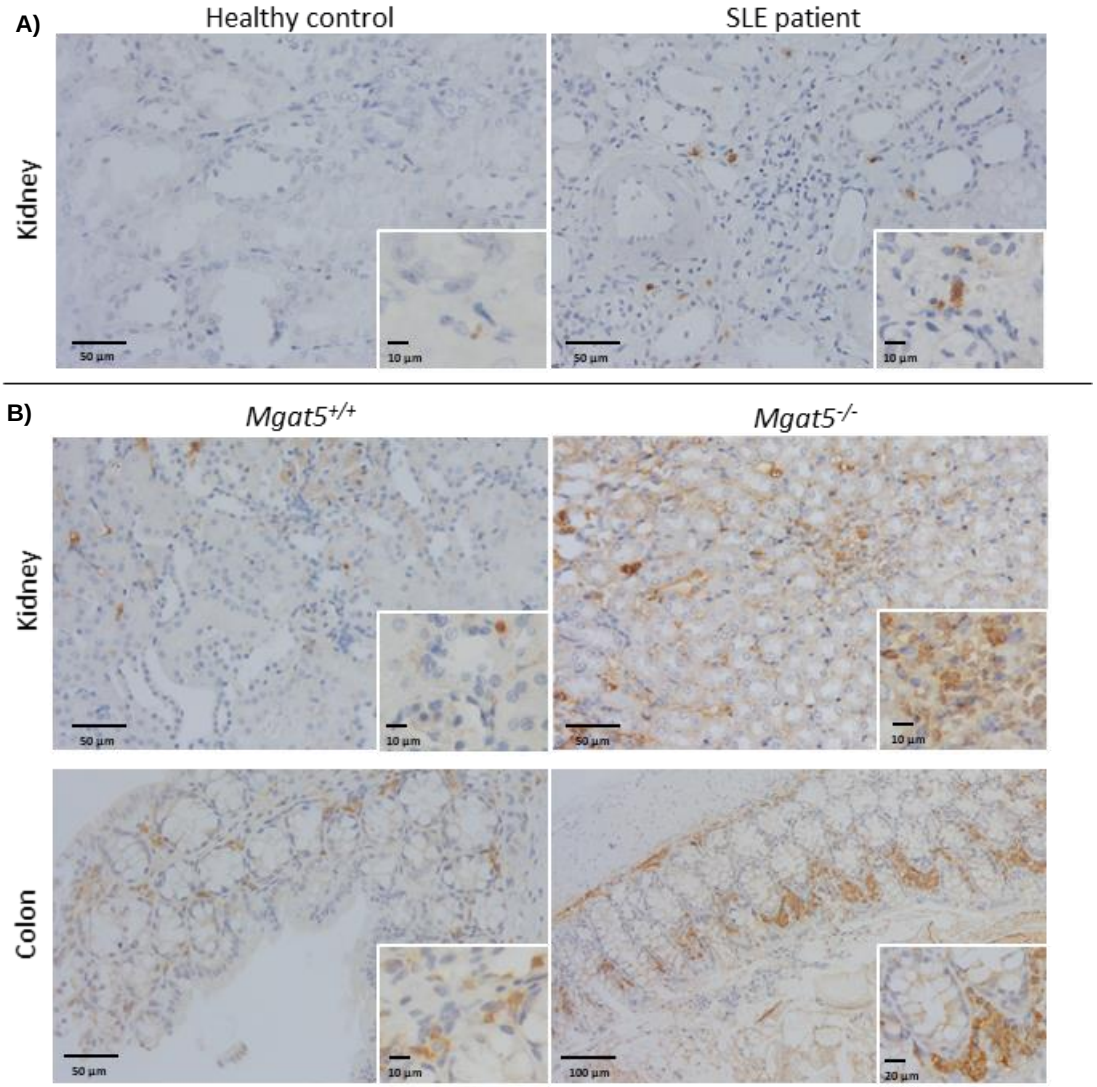

Figure 10 – Immunohistochemistry for γδT cells in kidney samples of SLE patients and kidney and colon samples of an immune-mediated mouse model. A) Immunohistochemistry for γδT cells in kidney samples of healthy control and SLE patient, where it is showed a clear infiltration of γδT cells in SLE patient compared with the control (n=2). Image magnification: 200x (400x inserts). **B)** Immunohistochemistry for γδT cells in kidney and colon samples of aging *Mgat5*$^{-/-}$ and WT mice, where it is also showed an increased infiltration of γδT cells in both organs from the KO mice (n=2). Image magnification for kidney and colon, respectively: 200x (400x inserts) and 100x (200x inserts).

2. Characterization of the glycophenotype of γδT cells at the periphery

Given the *in situ* accumulation of γδT cells in autoimmune environment, we questioned whether this cellular imbalance would also be reflected at the periphery of SLE patients. We have isolated PBMCs from SLE patients with and without LN (SLE LN and SLE nLN), as well as healthy individuals (Healthy) and analysed the frequencies of γδT cells on each group (Figure 11). No differences were observed among healthy and SLE (nor either between SLE patients with and without LN).

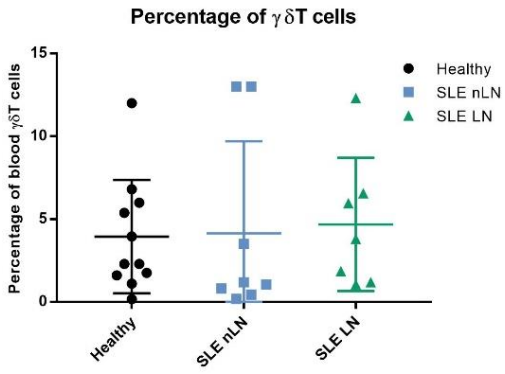

Figure 11 - Percentage of γδT cells in the peripheral blood of healthy controls, SLE patients without lupus nephritis (SLE nLN) and SLE patients with lupus nephritis (SLE LN), assessed by flow cytometry. The results show similar values in the analysed groups (n=11 healthy, n=8 SLE nLN and n=7 SLE LN). Each dot represents one individual biological sample, presented as mean with standard deviation (mean ± SD). Statistical significance was assessed by Kruskal-Wallis test with Dunn's test for multiple comparisons, although without statistical significance in any analysis performed (significant *p*-value ≤ 0.05).

Despite the absence of differences in the frequencies of cells, we characterized the glycosylation profile of γδT cells. To do so, we have accessed RNA-seq public data of human T cell subsets and compared the transcriptional levels of selected glycogenes comparing peripheral blood γδT cells and naïve $CD4^+$ T cells (Figure 12). In these samples, we showed that the transcript levels of *ALG3*, *DPAGT1*, *MAN1A1*, *MGAT1*, *MGAT2* and *MGAT5* genes were the highest in γδT cells in comparison with naive $CD4^+$ T cells. Additionally, in γδT cells we have observed a 2-fold or more increased expression of *DPAGT1*, *MAN1A2*, *MGAT1* and *MGAT2* genes, suggesting a strong dependency of γδT cells on the expression of proteins codified by these genes.

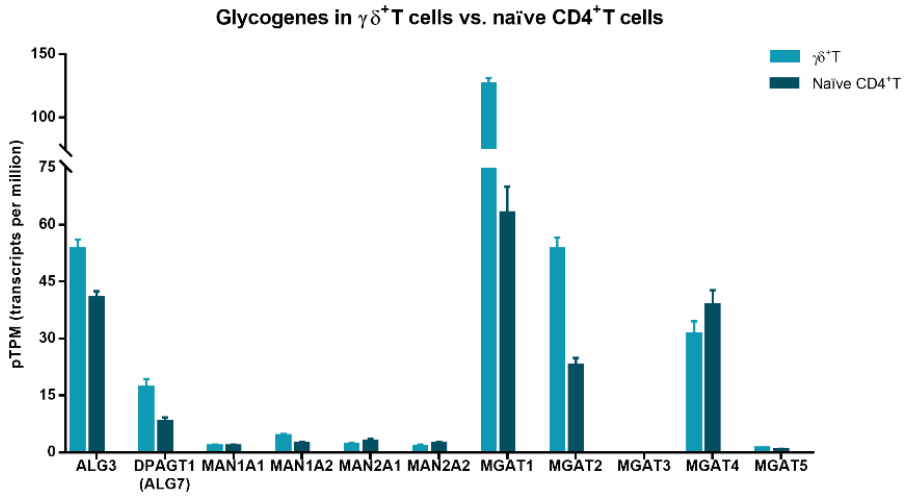

Figure 12 – Transcription levels of different glycogenes in γδT cells and naïve CD4+T cells collected from RNA-seq analysis of healthy human blood samples from the database "The human Protein Atlas - Blood Atlas". pTPM values, transcripts per million, give a quantification of the gene abundance which is comparable between different genes and samples. Alpha-1,3-Mannosyltransferase (*ALG3*) and dolichyl-phosphate *N*-acetylglucosaminephosphotransferase 1 (*DPAGT1*) genes that encode respectively $Man_5GlcNAc_2$-PP-Dol alpha-1,3-mannosyltransferase and *N*-acetylglucosamine-1-phosphate transferase (G1PT) involved in the synthesis of Dol-P. The remaining genes are mentioned above. Bars represent the mean ± SD.

We next analysed the glycophenotype of blood γδT cells from SLE patients and healthy controls (Figure 13). The γδT cells from SLE patients showed an increased expression of high-mannose *N*-glycans (detected by GNA binding) that accompanied the severity of the disease, whereas SLE LN patients display the highest levels of GNA reactivity. In accordance, it was also showed a reduction of branched *N*-glycans (L-PHA binding) in γδT cells from SLE LN patients (Figure 13). Interestingly, in SLE nLN the L-PHA mean value were slightly higher than the controls, although in general the L-PHA values were lower than the controls. Moreover, we also observed an increased expression of α-2,6 sialic acid (detected by SNA) in γδT cells from SLE patients, although with similar levels between healthy controls and SLE LN.

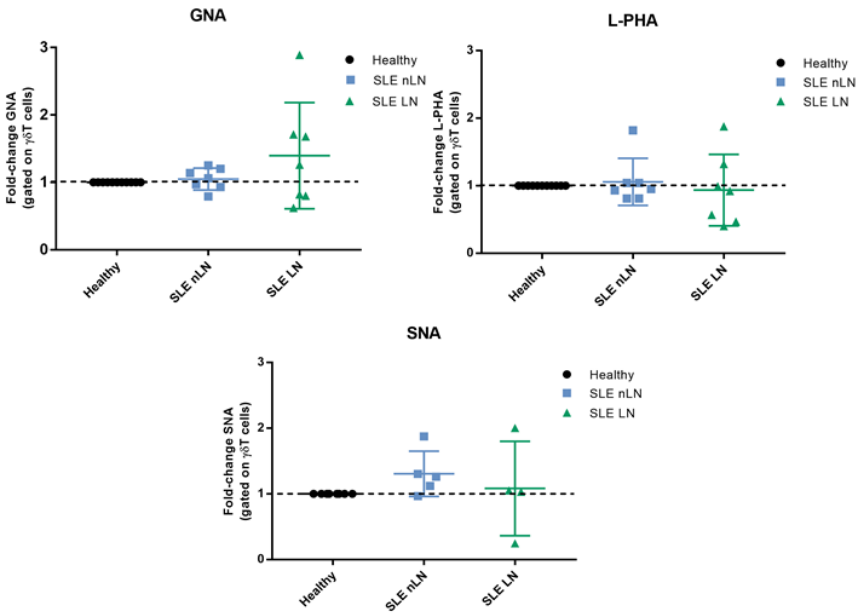

Figure 13 – Glycosylation profile of γδT cells in peripheral blood of healthy controls, SLE patients without lupus nephritis (SLE nLN) and SLE patients with lupus nephritis (SLE LN), assessed by flow cytometry. Fold-change of the MFI of high-mannose *N*-glycans (GNA), β1,6-branched *N*-glycans (L-PHA) and α-2,6 sialic acid (SNA) in γδT cells (n=11 healthy, n=8 SLE nLN and n=7 SLE LN, with exception on SNA staining n=9 healthy, n=6 SLE nLN and n=4 SLE LN). The results showed increased high-mannose *N*-glycans with the disease severity, as well as increased expression of α-2,6 sialic acid in SLE patients. In γδT cells of SLE LN was observed a slight reduction of branched *N*-glycans. The similar for SLE nLN, despite the higher mean value compared with the controls. Each dot represents one individual biological sample, presented as mean ± SD. All the results were normalized to the corresponding MFI of healthy control of each experiment. Statistical significance was assessed by Kruskal-Wallis test with Dunn's test for multiple comparisons, although without statistical significance in any analysis performed (significant *p*-value ≤ 0.05).

Additionally, we evaluated the glycosylation profile of IL-17-producing γδT cells (γδ17T cells) from SLE patients. We found a decreased of L-PHA/GNA ration in γδ17T cells from SLE LN patients, and similar levels between healthy and SLE nLN (Figure 14).

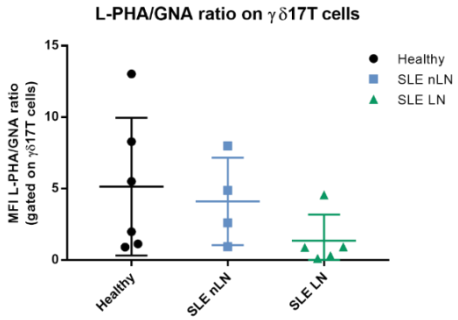

Figure 14 - Glycosylation profile of a subset of γδT cells in peripheral blood of healthy controls, SLE patients without lupus nephritis (SLE nLN) and SLE patients with lupus nephritis (SLE LN), assessed by flow cytometry. The L-PHA:GNA ratio was calculated as the ratio of the MFI of L-PHA and GNA levels gated in IL-17-producing γδT cells, showing a decreased ratio in SLE LN patients and similar levels between healthy and SLE nLN (n=7 healthy, n=4 SLE nLN and n=5 SLE LN). Each dot represents one individual biological sample, presented as mean ± SD. Statistical significance was assessed by Kruskal-Wallis test with Dunn's test for multiple comparisons, although without statistical significance in any analysis performed (significant *p*-value ≤ 0.05).

Here we demonstrated, for the first time, a unique and altered glycosylation profile of circulating γδT cells from SLE patients, suggesting β1,6 branching *N*-glycans in γδT cells as an important regulator of activity and function of these cells in SLE.

3. Characterization of the glycosylation profile of γδT cells in different tissues and diseases environments

In order to characterize the γδT cells' glycophenotype in different environments we selected organs from *Mgat5*$^{-/-}$ (KO) mouse model with 8 weeks- and 15 months-old. This mouse model lacks the *Mgat5* gene that encodes the enzyme responsible for the production of β1,6-GlcNAc branched *N*-glycans (Figure 15 and Figure 16). As a control we used the *Mgat5*$^{+/+}$ mice (WT) with 8 weeks- and 15 months-old. Previous studies from our group have shown that mice lacking GnT-V enzyme (encoded by *Mgat5* gene) display an increased susceptibility to autoimmune-mediated disease, such as SLE (not published) and IBD[144]. Thus, and knowing that γδT cells are also mediators of the immune response, but with an unclear role in the different tissues, we hypothesized that different glycoprofiles of γδT cells in different tissues might constitute another layer of regulation of this cell subset.

Our results showed that γδT cells from 8 weeks-old *Mgat5*$^{-/-}$ mice exhibited an increased activation (by CD69 expression, an earlier activation marker) both in colon, kidney and in lung, although in spleen there are no differences in activation (Figure 15A). The lack of branched *N*-glycans was confirmed by L-PHA staining, where γδT cells from KO mice showed an absence of L-PHA reactivity. Moreover, these cells display increased expression of high-mannose *N*-glycans (by GNA binding) in colon, kidney and lung, although no differences in spleen were observed. Interestingly, in KO mice, γδT cells showed an increased expression of polylactosamine structures (by LEL binding) in colon, kidney and in lung and a decreased in spleen, as well as an increase in α-2,6 sialic acid residues in all organs (by SNA binding). Additionally, we evaluate the expression of intracellular cytokines in γδT cells, between WT and KO mice, after PMA/Ion stimulation (Figure 15B). Interestingly, we verified an increased expression of intracellular IL-17 in all KO-organs, compared with the WT mice. We also observed a slight increase of intracellular IFNγ in γδT cells only from the KO-kidney.

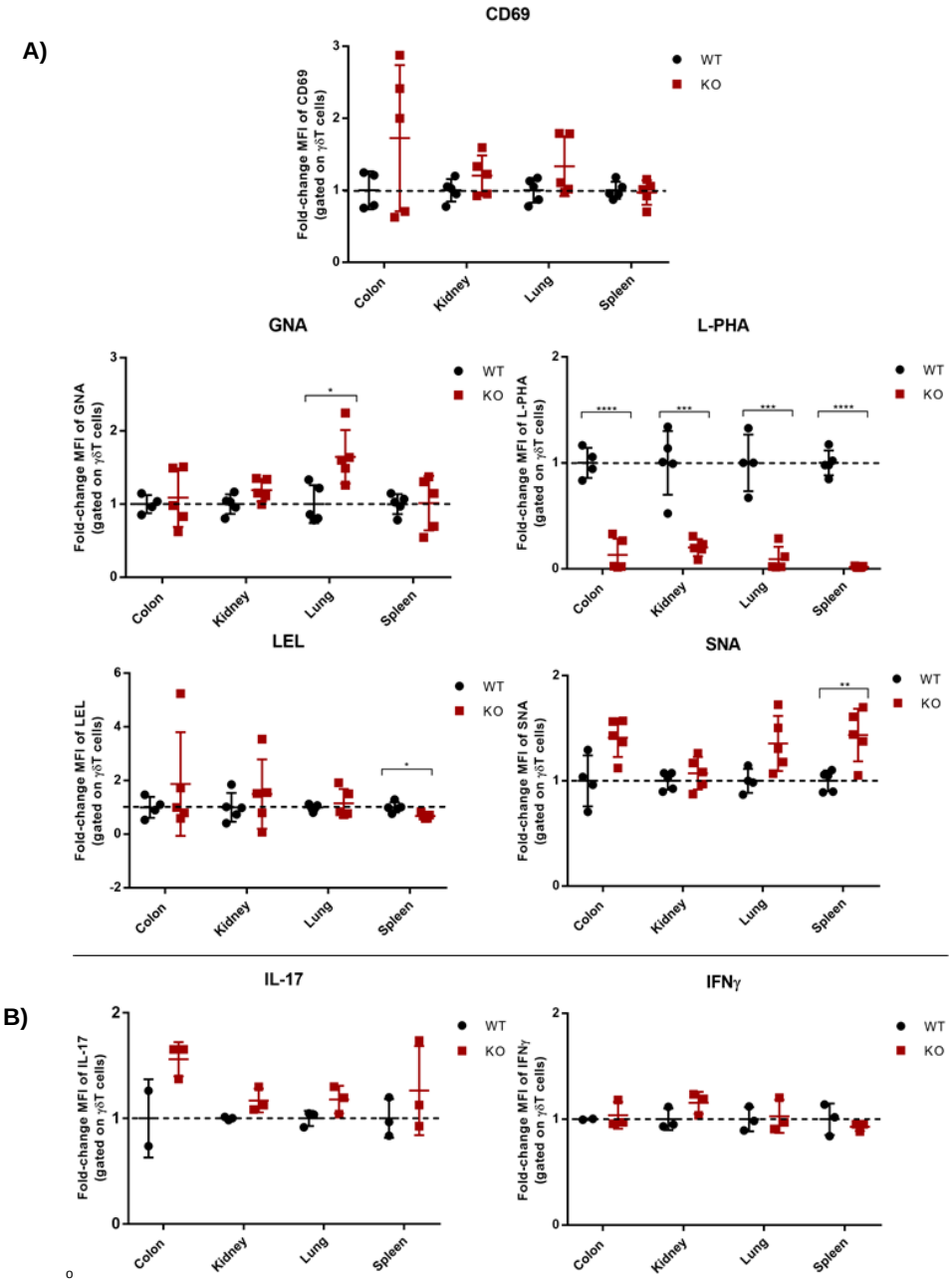

Figure 15 – Activation, glycosylation profile and cytokines of γδT cells in the colon, kidney, lung and spleen from 8 weeks-old *Mgat5*$^{-/-}$ mice (KO) compared with 8 weeks-old WT mice, assessed by flow cytometry. A) Fold-change of the MFI of the activation marker CD69 and different lectins GNA, L-PHA, LEL and SNA in γδT cells of colon, kidney, lung and spleen from KO compared with WT mice. The results showed increase activation (CD69), high-mannose *N*-glycans (GNA) and polylactosamine structures (LEL) in γδT cells of colon, kidney and lung from the KO mice, and a neglected expression of branched *N*-glycans (L-PHA) in γδT cells of all organs from the KO mice. The α2,6-sialic acid (SNA) expression was increased in γδT cells from all KO organs. (n=5 WT, n=5 KO) **B)** Fold-change of the MFI of intracellular IL-17 and IFNγ in γδT cells of colon, kidney, lung and spleen from KO compared with WT mice, after PMA/Ion stimulation. The results showed increased IL-17 expression in γδT cells of all organs from KO mice and increased expression of IFNγ in γδT cells only of the KO kidney (n=3 WT, n=3 KO). All the results were normalized to the mean value of MFI from WT mice of each experiment, and the outliers were removed. Each dot represents one individual biological sample, presented as mean ± SD. Statistical significance was assessed by multiple t-test with Holm-Sidak method, **p*-value ≤ 0.05, ** *p*-value ≤ 0.01, *** *p*-value ≤0.001, **** *p*-value ≤0.0001.

Evidences from our group showed that 15 months-old *Mgat5*-KO mice develop autoimmune-like syndrome associated with aging (not published). So, we have analysed the glycoprofile of these aging mice (Figure 16). The results showed that γδT cells from *Mgat5*$^{-/-}$ mice display an increased CD69 expression in colon, kidney and lung, with the lowest levels in the lung. The absence of branched *N*-glycans was also confirmed by neglected detection of L-PHA in γδT cells from all analysed KO-organs. Through GNA binding, it was observed a remarkable increase of high-mannose *N*-glycans in γδT cells from colon and specially in kidney, although in lung these cells showed no major differences in the content of mannosylated glycans. Regarding polylactosamine (LEL binding) and α-2,6 sialic acid (SNA binding), it was observed an increased expression of these two structures in γδT cells from colon and kidney of KO mice and no differences of both for γδT cells in lung of KO mice compared with the WT.

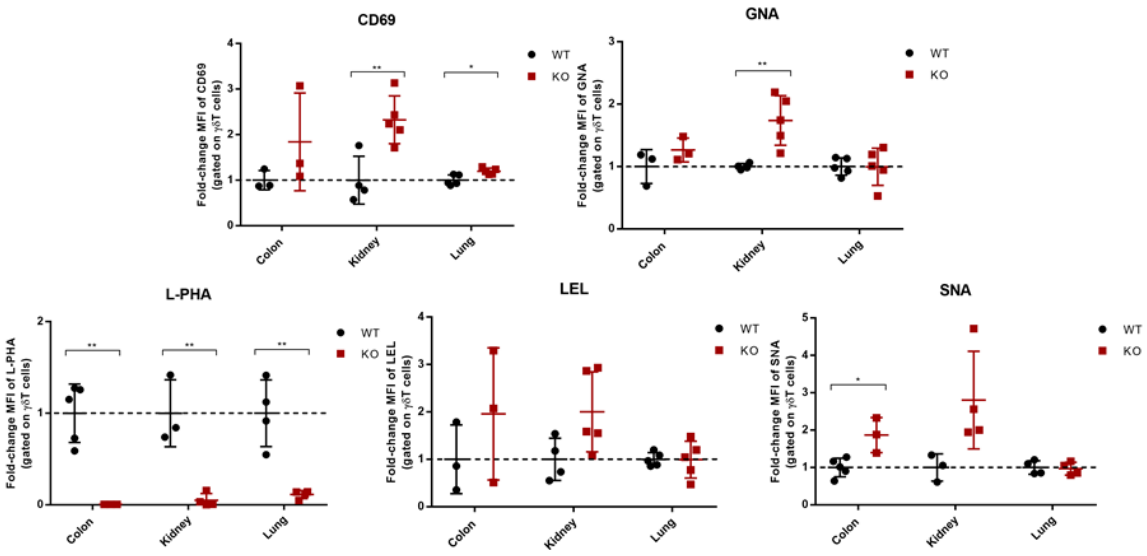

Figure 16 – Activation and glycosylation profile of γδT cells in the colon, kidney and lung from 15 months-old *Mgat5*$^{-/-}$ mice (KO) compared with 15 months-old WT mice, assessed by flow cytometry. Fold-change of the MFI of the activation marker CD69 and different lectins GNA, L-PHA, LEL and SNA in γδT cells of colon, kidney and lung from KO compared with WT mice. The results showed increase activation (CD69) in γδT cells of all analysed organs from the KO mice, as well as increased high-mannose *N*-glycans (GNA) in the colon and kidney of KO mice. All the organs from the KO mice display neglected expression of branched *N*-glycans (L-PHA) in γδT cells. Both polylactosamine (LEL) and α-2,6 sialic acid (SNA) structures were highly expressed in γδT cells of colon and kidney from the KO mice. (n=5 WT, n=5 KO, the outliers in each analysis were removed). All the results were normalized to the mean value of MFI from WT mice of each experiment, and the outliers were removed. Each dot represents one individual biological sample, presented as mean ± SD. Statistical significance was assessed by multiple t-test with Holm-Sidak method, **p*-value ≤ 0.05, ** *p*-value ≤ 0.01.

Additionally, we have evaluate the intracellular cytokines of γδT cells from mesenteric lymph nodes (MLNs) after PMA/Ion stimulation, as well as the concentration of IL-17 and IFNγ in the supernatants of renal explants from the aging mice (Figure 17). Interestingly, in the lymphoid organ from the KO mice we verified a decrease in intracellular IL-17 and

IL-22 of γδT cells comparing with the WT mice (Figure 17A). In renal explants from KO mice, it was showed a slight increase on the concentration of both IL-17 and IFNγ cytokines (Figure 17B).

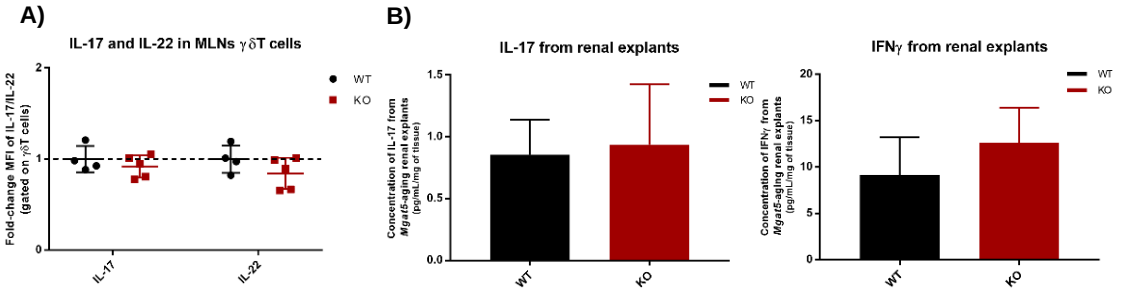

Figure 17 – Cytokines in mesenteric lymph nodes (MLNs) and kidney from 15 months-old *Mgat5*$^{-/-}$ (KO) mice compared with WT mice. A) Fold-change of the MFI of intracellular IL-17 and IL-22 in γδT cells, after PMA/Ion stimulation of γδT cells from the MLNs of *Mgat5*-aging mice, showing decreased expression of both cytokines in γδT cells from the KO compared with WT mice (n=5 WT, n=5 KO, the outliers in each analysis were removed). The results were normalized to the mean value of MFI from WT mice. Each dot represents one individual biological sample, presented as mean ± SD. **B)** Concentration of IL-17A and IFNγ in the supernatants of renal explants from *Mgat5*-aging mice, expressed as pg/mL/mg of tissue, (n=5 WT, n=5 KO). Showing a slight increase in IL-17A and IFNγ in the KO compared with WT mice. Bars represent mean ± SD. Statistical significance was assessed by t- test with Mann-Whitney test, although both analysis do not reveal any significance statistical (significant *p*-value ≤ 0.05).

4. Remodelling the glycosylation profile of γδT cells: impact in their activation and cytokine profile

At this point, we have demonstrated a unique and altered γδT cells' glycosylation profile, associated with increased γδT cell kidney infiltrates in SLE patients, that may result in an altered γδT cell immune response, as observed in mouse model absent in tetra-antennary *N*-glycans. Therefore, we hypothesized that a specific (abnormal) glycoprofile might define specific functions of γδT cells associated with a pro-inflammatory phenotype. To address this point, we performed an *in vitro* glycosylation reprogramming of γδT cells, further evaluating its functional impact in the activity of the cells associated with SLE immunopathogenesis, through flow cytometry.

Since γδT cells biology has only been explored more recently, there is a lack of information regarding *in vitro* cultures, as well as robust markers, kinetic of activation and cytokines-specific profiles. This brought another layer of complexity to the work, which led us to perform an optimization assay to select the ideal culture time and activation marker for γδT cells. Therefore, isolated γδT cells from healthy donors' buffy coats were stimulated with anti-CD3/CD28 mAb and treated with kifunensine (KF), a widely used and potent mannosidase I inhibitor that inhibit the synthesis of complex *N*-glycans. It is important to refer that γδT cells express CD3 and CD28 receptors at cell surface and depends on them for their activation, survival and proliferation, as so we used anti-CD3 and anti-CD28 mAb for γδT cells stimulation in this *in vitro* experiment[159–161]. Since CD69, CD25 and HLA-DR are the most described activation markers for T and γδT cell activation, we select these three cell surface markers and four timepoints (7 h, 24 h, 48 h and 72 h) to evaluate the glycosylation profile and activation of γδT cells treated with KF (KF-treated γδT cells) and non-treated cells (Basal)[162–166]. Our results showed a clear increased activation of KF-treated γδT cells (increased reactivity to CD25 and HLA-DR) for all timepoints when comparing to non-treated γδT cells (basal) (Figure 18A). CD69 overexpression was only observed at 48 h. The CD25 was selected as an activation marker to pursue, once fluorochrome attached to this was preferable for the flow cytometry mixes of *in vitro* ablation of complex *N*-glycans. Importantly, from the 24 h to 72 h timepoints it was observed a substantial shift in γδT cells glycophenotype that was characterized by an increase in high-mannose *N*-glycans expression (by GNA binding) and a decrease in branched *N*-glycans expression (by L-PHA binding) (Figure 18B). The 24 h timepoint was selected as the most suitable timepoint to proceed in which KF-treated γδT cells presented high-mannose *N*-glycans accumulation and diminished branched *N*-glycans, with differences in the activation marker.

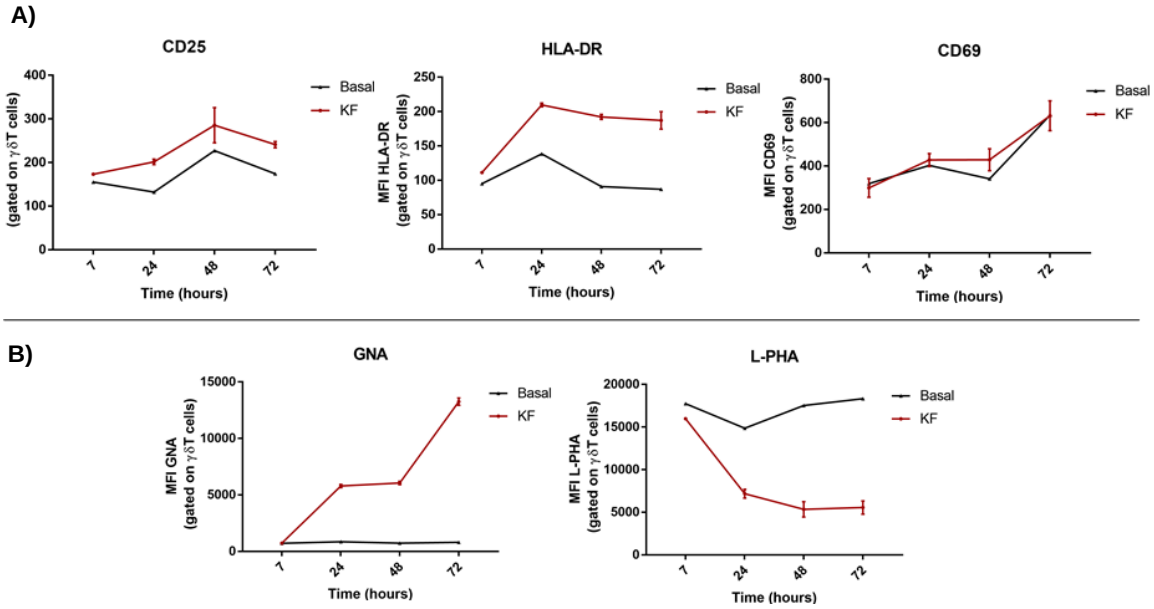

Figure 18 – *In vitro* re-program of γδT cells glycosylation profile: Optimization assay to select the ideal culture time and activation marker for *in vitro* ablation of complex *N*-glycans, through flow cytometry. A) Activation profile (MFI) of KF-treated γδT cells (KF) and non-treated cells (basal) evaluated with different activation markers - CD25, HLA-DR and CD69 - at different timepoints (7 h, 24 h, 48 h and 72 h). The results showed increased activation in all timepoints of KF-treated γδT cells by CD25 and HLA-DR reactivity, although CD69 overexpression was only observed at 48 h. **B)** Glycosylation profile (MFI) of KF-treated γδT cells (KF) and non-treated cells (basal), at different timepoints (7 h, 24 h, 48 h and 72 h). The results showed increased expression of high-mannose *N*-glycans (by GNA binding) and decreased expression of branched *N*-glycans (by L-PHA binding) of KF-treated γδT cells, in the last three timepoints. The selected activation marker and culture time were CD25 and 24 h, respectively. The results are presented as mean ± SD. (Basal n=1; KF n=1, technical duplicates) Statistical analysis was not assessed since the number of samples is very few.

After this optimization, γδT cells isolated from PBMCs of three healthy donors' buffy coats (n=3) were cultured as previous described, for 24 h. At the end of the culture time, the cellular glycophenotype, activation status and cytokines profile were analysed by flow cytometry (Figure 19). Accordingly to what was observed above, KF-treated γδT cells present higher levels of surface CD25, suggesting a more activation status comparing to non-treated cells (Figure 19A). This was accompanied with a significant decrease in the expression of branched *N*-glycans (by L-PHA binding) and a substantial increased expression of mannose structures (by GNA binding), compared with non-treated cells. In addition, we also found a significant decrease in α-2,6 sialic acid (lower SNA binding) in the γδT cells. These results suggest that lack of *β*1,6-GlcNAc structures resulted in a more autoreactive γδT cell response. Furthermore, we also evaluated the intracellular cytokines expression, as well as the percentage of cytokines-producing γδT cells, trough intracellular staining (Figure 19B). Interestingly, after CD3/CD28 stimulation we observed that the removal of complex *N*-glycans of γδT cells (KF-treated γδT cells) revealed an increased expression of pro-inflammatory cytokines, such as IL-17 and IFNγ, and a decrease on the production of anti-inflammatory cytokine, IL-10. This was

accompanied by an increased percentage of IL-17- and IFNγ-producing γδT cells and a lower percentage of IL-10-producing γδT cells when compared with non-treated cells.

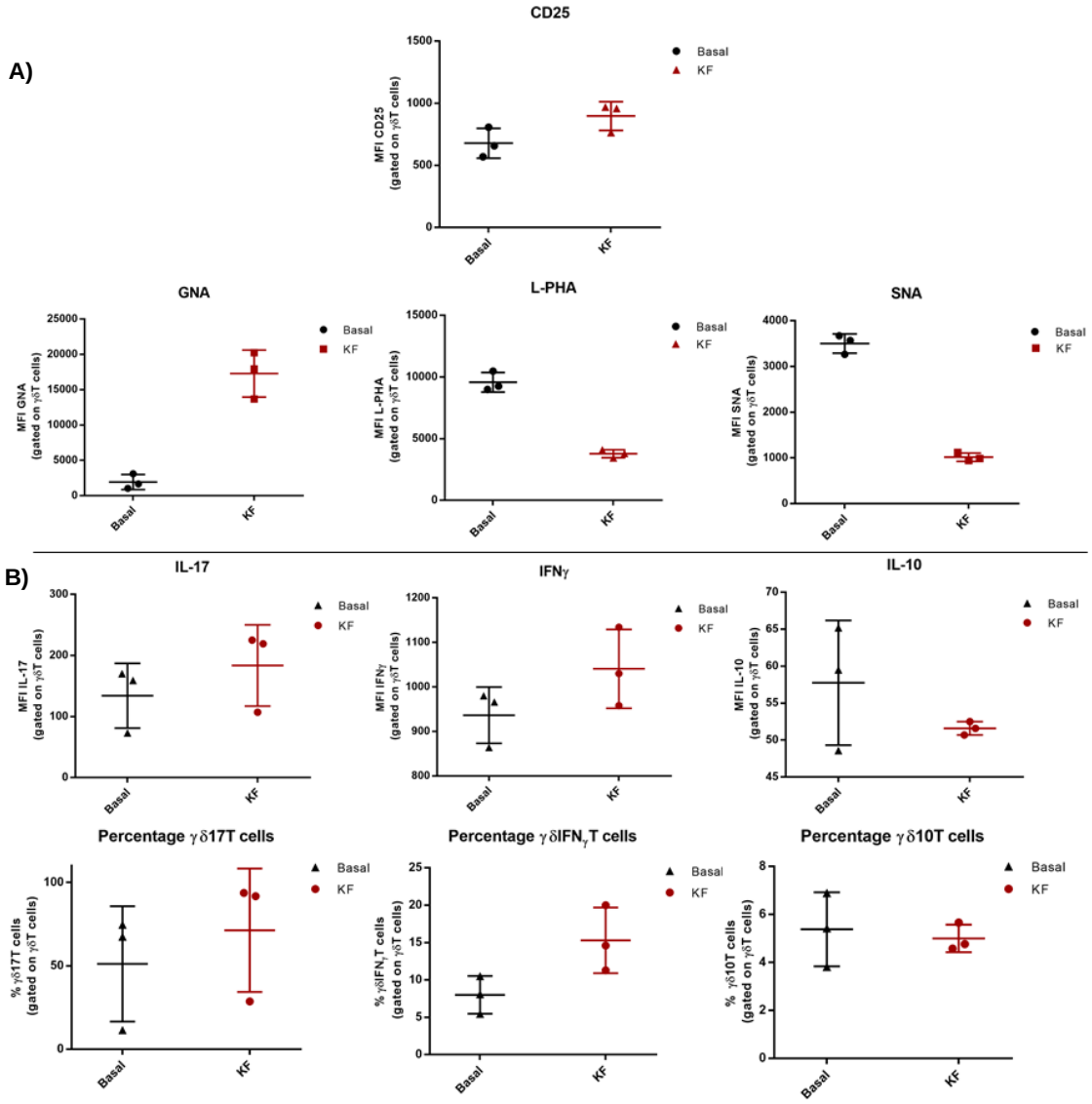

Figure 19 – Activation, glycosylation and cytokine profile of KF-treated γδT cells compared with non-treated cells, assessed by flow cytometry. A) Activation and glycosylation profile (MFI) of KF-treated γδT cells (KF cells) and non-treated cells (basal cells). It is showed enhanced activation of KF cells (CD25), a decreased of branched *N*-glycans (L-PHA) and α-2,6 sialic acid (SNA), as well as an increase of high-mannose *N*-glycans (GNA staining). **B)** Cytokine profile (MFI) of KF-treated γδT cells (KF cells) and non-treated cells (basal). The results showed an increased expression of pro-inflammatory cytokines (IL-17 and IFNγ) and a decreased expression of IL-10 cytokine. The percentage of IL-17- and IFNγ-producing γδT cells (γδ17T and γδIFN$_\gamma$T cells) was higher in KF cells, and the opposite for IL-10-producing γδT cells (γδ10T cells). Each dot represents one individual biological sample (Basal n=3, KF n=3), presented as mean ± SD. Statistical significance was assessed by t-test with Mann-Whitney test, although without statistical significance in any analysis performed (significant *p*-value ≤ 0.05).

Taking into consideration that KF inhibits the synthesis of complex *N*-glycans in all proteins, we have confirmed whether γδT cell receptor (γδTCR) could be a target of this glyco-modulation. For that, we verified the presence of putative *N*-glycosylation sites *in silico* in γδT cell. Accordingly to the Uniprot database, the γδTCR display 6 to 8 *N*-

glycosylation sites, represented in Table 3. In line with this, it was observed a shift in the L-PHA Western blot band in the molecular weight of γδTCR (18 kDa) upon KF treatment (Figure 20). This preliminary result support γδTCR as a possible target of glycosylation modification potentially implicated in the regulation of activity and signalling of γδT cells, further corroborated by the high number of putative *N*-glycosylation sites.

Table 3 – *N*-glycosylation sites of γδTCR. The data was obtained from the UniProt database.

δ chain	**N-glycosylation sites**
T cell receptor delta constant	2
T cell receptor delta variable 1	0
T cell receptor delta variable 2	0
T cell receptor delta variable 3	0
γ chain	***N*-glycosylation sites**
T cell receptor gamma constant 1	4
T cell receptor gamma constant 2	5
T cell receptor gamma variable 2	0
T cell receptor gamma variable 3	1
T cell receptor gamma variable 4	1
T cell receptor gamma variable 5	1
T cell receptor gamma variable 8	0
T cell receptor gamma variable 9	0
TOTAL in γδTCR	**6 to 8**

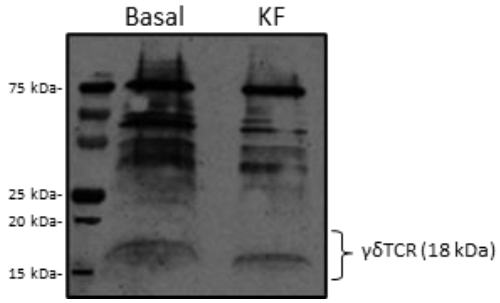

Figure 20 – Western blot analysis from sorted γδT cells treated with KF and non-treated cells (Basal). Proteins were stained with L-PHA lectin to evaluate the expression of branched *N*-glycans on γδTCR. Positive reaction was observed in a protein band with the same size as γδTCR (18 kDa).

5. *In vivo* remodelling of the glycosylation profile modulates γδT cells activity and cytokine response

Finally, we went to validate our *in vitro* observations using a glycoengineered animal model absence of complex *N*-glycans. For that, we used a mouse model lacking the *Mgat2* gene in *Rag1*-expressing cells – $Rag1^{Cre/+}Mgat2^{fl/f}$ - a conditional knock-out in which B and T cells lack complex *N*-glycans[167]. The γδT cells from the spleen were sorted, cultured for 24 h with anti-CD3/CD28 mAb stimulation and stained for flow cytometry (Figure 21). For controls we used the $Rag1^{+/+}Mgat2^{fl/f}$ mice (*Mgat2*-WT mice). As expected, we observed through CD69 staining an hyperactivation of γδT cells from knock-out mice, as well as an increased binding of GNA, SNA and LEL (Figure 21A). We further demonstrated an increase of mannose-enriched *N*-glycans (high-mannose and hybrid) associated with increased activation of γδT cells. In addition, we also demonstrated a more pro-inflammatory response of γδT cells from the KO mice, where is observed an increased expression of intracellular IL-17 and IFNγ and a decreased expression of IL-10 (Figure 21B).

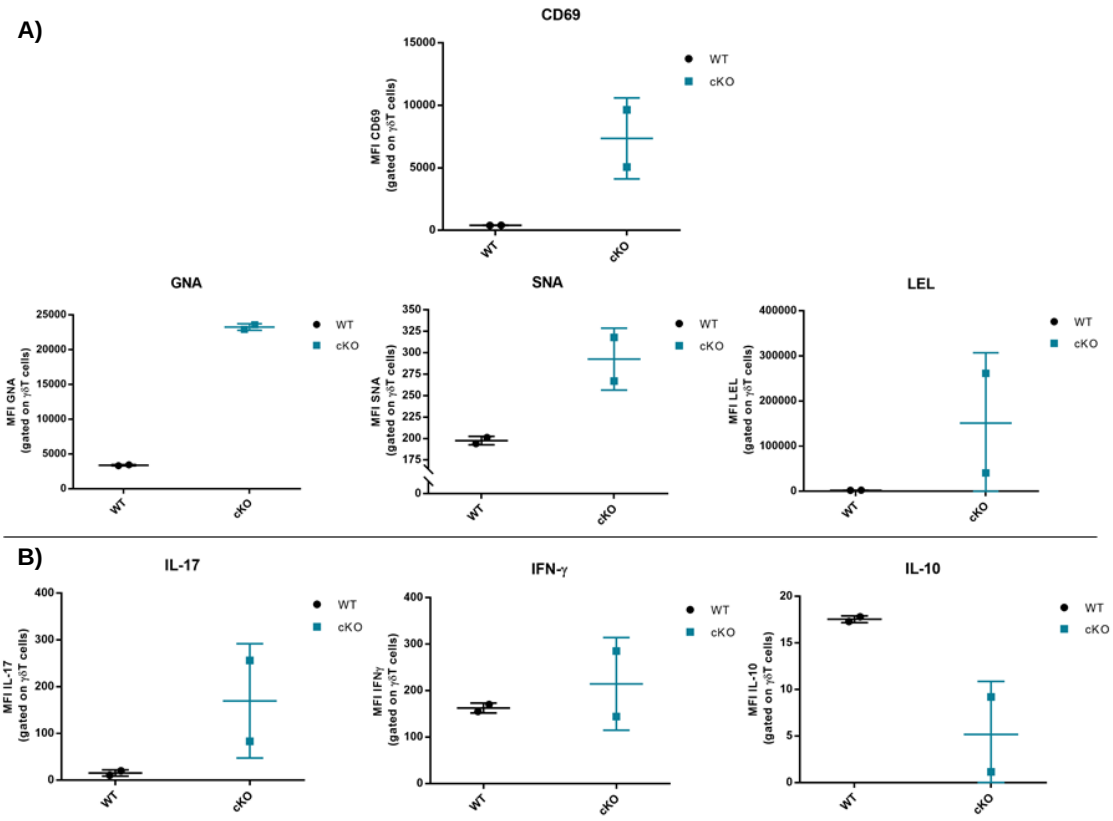

Figure 21 – Activation, glycosylation profile and intracellular cytokines in sorted γδT cells of spleen from *Rag1*$^{Cre/+}$*Mgat2*$^{fl/f}$ mice, conditional knock-out (cKO) compared with WT mice, assessed by flow cytometry. A) Activation and glycosylation profile (MFI) of γδT cells from KO compared with WT mice. The results showed enhanced activation (CD69), as well as increased high-mannose *N*-glycans (GNA), polylactosamine structures (LEL) and α-2,6 sialic acid (SNA) in γδT cells from the KO mice. **B)** Intracellular cytokines (MFI) expression of γδT cells from KO compared with WT mice, showing increased expression of IL-17 and IFNγ in γδT cells and a decrease of IL-10 expression. Each dot represents one individual biological sample (n=2 WT, n=2 KO), presented as mean ± SD. Statistical analysis was not assessed since the number of samples is very few.

Overall, our data suggest that the lack of complex *N*-glycans in γδT cells, either through an enzymatic or genetic approach, modulate γδT cell activity and function, where a more autoreactive and pro-inflammatory γδT cell response is observed.

6. Characterization of the glycan-binding receptors from γδT cells

Another factor that influences the activation and signalling of immune cells is the expression of glycan-binding proteins at cell surface, such as CTLs, capable of sensing glycans from the environment[128]. Despite CTLs being canonically expressed by myeloid cells such as dendritic cells and macrophages, we hypothesized that innate-like γδT cells could potentially express these receptors given their ability to sense, process and present antigens[168]. Up to now, only a few CTLs were described in γδT cells[169,170]. Therefore, we aimed to characterize the glycans receptors at γδT cell surface. Interestingly, we verified through an *in silico* analysis that γδT cells from healthy donors constitutively express genes that encode different CTLs, including *CLEC7A* (Dectin-1), *CLEC4L* (DC-SIGN) and *CLEC4E* (Mincle) genes, although no detection of *MRC1* (MR) and *CLEC6A* (Dectin-2) (Figure 22).

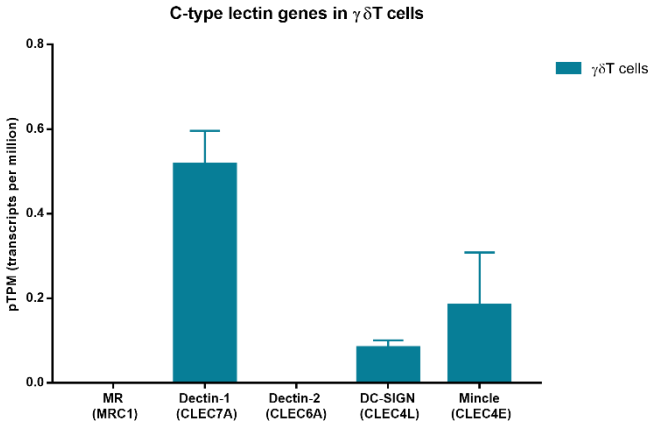

Figure 22 – Transcription levels of different C-type lectin genes in γδT cells collected from RNA-seq analysis of healthy human blood samples from the database "The human Protein Atlas - Blood Atlas". pTPM (transcripts per million) values give a quantification of the gene abundance which is comparable between different genes and samples. Bars represent the mean ± SD.

To validate these *in silico* observations, we analysed the expression of these receptors on the surface of γδT cells by flow cytometry. As so, we evaluate the expression of one and/or two CTLs in the peripheral blood of SLE patients, as well as in autoimmune-susceptible mouse model. Blood peripheral γδT cells from SLE patients displayed an increased expression of DC-SIGN and MR levels compared with healthy controls, more evident in DC-SIGN expression (Figure 23A). Interestingly, the expression of these CTLs seem to be associated with the specific glycoprofile of γδT cells, since in SLE patients the γδT cells with lower GNA levels present the highest expression of MR, whereas γδT cells with the highest GNA levels display increased levels of DC-SIGN (Figure 23B).

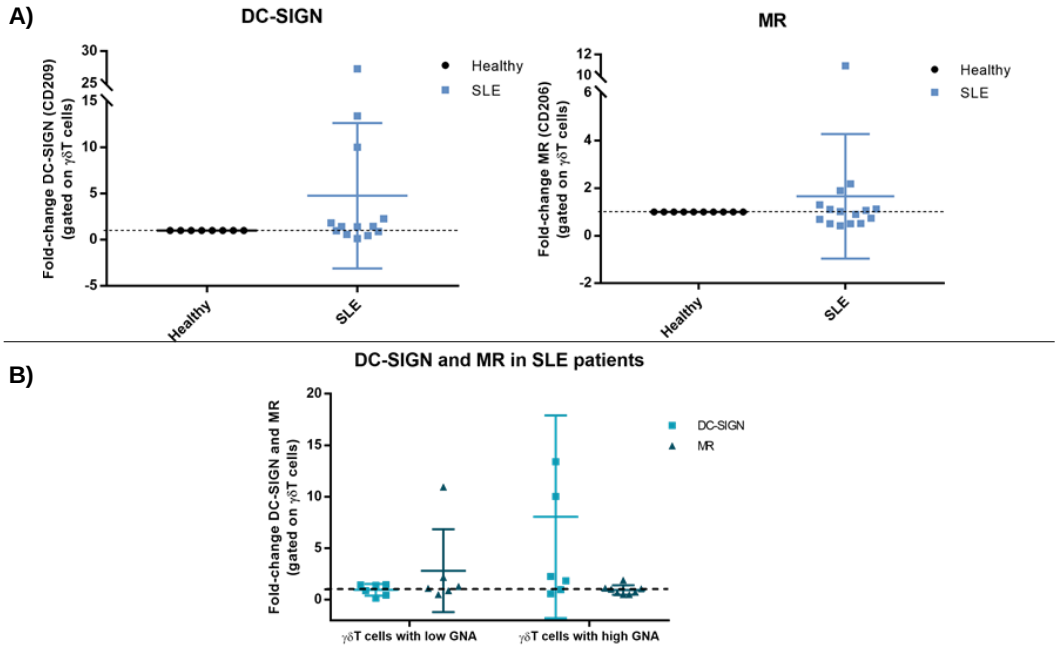

Figure 23 – Expression of C-type lectins in peripheral blood γδT cells from SLE patients (with and without lupus nephritis) compared with healthy controls, assessed by flow cytometry. A) Fold-change of the MFI of DC-SIGN (CD209) and MR (CD206) on gated γδT cells from SLE patients and healthy controls. The results revealed an increase of both CTLs in SLE patients. **B)** Fold-change of MFI of DC-SIGN and MR gated on γδT cells with low or high expression of GNA from SLE patients. It is showed an increased expression of MR in γδT cells with low GNA and increased of DC-SIGN expression in γδT with high GNA. Each dot represents one individual biological sample, presented as mean ± SD (DC-SIGN n=8 healthy and n=13 SLE; MR n=10 healthy and n=15 SLE). The results were normalized to the corresponding MFI of healthy control of each experiment. Statistical significance was assessed by t-test with Mann-Whitney test, although without statistical significance in any analysis performed (significant *p*-value ≤ 0.05).

As a proof of concept, we analysed the expression of DC-SIGN and MR on KF-treated γδT cells and verified a substantial increase of DC-SIGN expression in KF-treated γδT cells compared with non-treated cells, although we did not observed differences in MR expression (Figure 24).

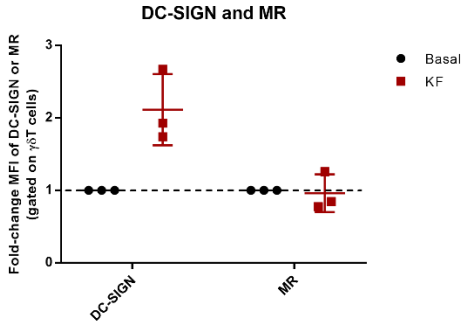

Figure 24 – Evaluation of the expression levels of C-type lectins in KF-treated γδT cells compared with non-treated cells, assessed by flow cytometry. Fold-change of the MFI of DC-SIGN (CD209) and MR (CD206) on gated γδT cells treated with KF (inhibitor of complex *N*-glycans synthesis) compared with non-treated cells (Basal). It is demonstrated an increased expression of DC-SIGN in KF cells, although no differences in MR expression. Each dot represents one individual biological sample (Basal n=3, KF n=3), presented as mean ± SD. Statistical significance was assessed by t-test with Mann-Whitney test, although without statistical significance in any analysis performed (significant *p*-value ≤ 0.05).

Regarding the 8 weeks-old *Mgat5*$^{-/-}$ mice (lacking β1,6-GlcNac branched *N*-glycans) there are no differences in MR expression when compared with WT mice (kidney, lung and spleen), although in the colon there is an increase in the mean of MR expression, due to the heterogeneity between animals (Figure 25A). Moreover, the aging *Mgat5*$^{-/-}$ mice displayed an increased expression of MR in γδT cells from all analysed organs (colon, kidney and lung) (Figure 25B).

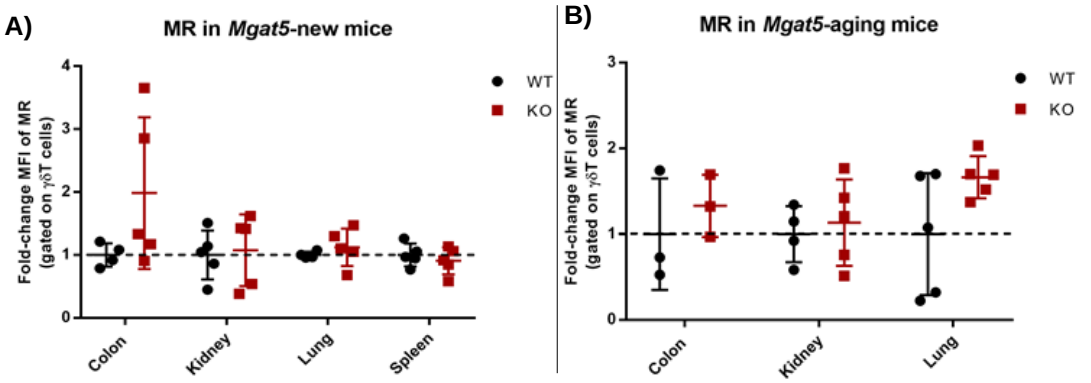

Figure 25 – Expression of MR in γδT cells of different organs from *Mgat5*$^{-/-}$ (KO) mice with 8 weeks-old (new) and 15 months-old (aging) compared with respective WT mice, assessed by flow cytometry. A) Fold-change of the MFI of MR on gated γδT cells of colon, kidney, lung and spleen from *Mgat5*$^{-/-}$ new mice and respective WT mice. The results showed increase MR expression in γδT cells of colon from KO mice. **B)** Fold-change of the MFI of MR on gated γδT cells of colon, kidney and lung from *Mgat5*$^{-/-}$ aging mice and respective WT mice, demonstrating an increase percentage of MR in γδT cells of all organs from the KO mice. All the results were normalized to the mean value of MFI from WT mice, (n=5 WT, n=5 KO, the outliers in each analysis were removed). Each dot represents one individual biological sample, presented as mean ± SD. Statistical significance was assessed by multiple t-test with Holm-Sidak method, although without statistical significance in any analysis performed (significant *p*-value ≤ 0,05).

Additionally, when we earlier blocked the glycosylation pathway, we observed a remarkable difference when comparing the *Mgat2*$^{-/-}$ cKO mice and WT, in which cKO revealed a significant increase expression of MR in γδT cells of spleen (Figure 26).

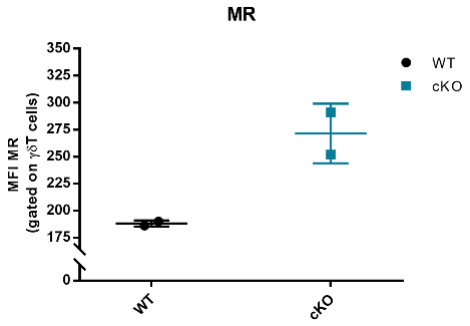

Figure 26 - Expression of MR in γδT cells of spleen from conditional *Mgat2*$^{-/-}$ (cKO) mice compared with respective WT, assessed by flow cytometry. MFI of MR on gated γδT cells of spleen. Showing increase MR expression in γδT cells from KO mice. Each dot represents one individual biological sample (n=2 WT, n=2 cKO), presented as mean ± SD. Statistical analysis was not assessed since the number of samples is very few.

Discussion

SLE is a heterogeneous and incurable autoimmune disease with an increase prevalence in women in their active period of life. The loss of immune tolerance is one of the triggers involved in SLE, although the cellular and molecular mechanisms responsible for the etiopathogenesis remains unknown[24]. *N*-glycosylation is a crucial mechanism that is inherent to all mammalian cells, where glycans and glycan-binding proteins interactions are crucial for homeostasis. Glycans play vital roles in cells, participating in a variety of important cellular mechanisms, such as cell-cell and cell-matrix interactions, as well as protein function and trafficking[109]. The breakdown of *N*-glycosylation pathway have therefore huge implications in the regulation of immune system, such as in T cell function and signalling, as well as in cancer and autoimmunity[115]. Interestingly, the γδT cells are a minor group of T lymphocytes abnormally expressed in SLE patients and described in other autoimmune diseases. In this thesis, we aim to unravel the impact of glycosylation alterations in γδT cells' activity and functions and its consequences in the immunopathogenesis of SLE.

Our *in situ* results demonstrated increased levels of γδT cells' infiltrates in paraffin kidney-biopsies from SLE patients, which was supported by other studies, for example the Vδ2 T cell was found accumulated in the kidney from SLE patients[85]. Similarly, we observed an accumulation of γδT cells in the organs from an autoimmune disease-susceptible mouse model (*Mgat5*$^{-/-}$ mice). At the periphery, when analysed the PBMCs of SLE patients, we verified a similar percentage of γδT cells compared to the healthy controls, which is in accordance with previous studies in which the γδT cells frequencies were almost the same or even diminished in SLE patients[85,171]. Together, an abnormal γδT cells *in situ* profile was demonstrated, suggesting that these cells are being recruited from peripheral blood infiltrating the kidney of SLE patients, and also observed in immune-mediated mouse model.

Since it was observed an *in situ* accumulation of γδT cells, we hypothesized whether an altered γδT cells' activity and function might be explained by an abnormal expression of *N*-glycans at the surface of these cells. We demonstrated *in silico* an increased expression of genes involved in the *N*-glycosylation pathway by γδT cells, suggesting a high dependency on G1PT, α-Man IIA, GnT-I and GnT-II enzymes, demonstrated by the high levels of *DPAGT1*, *MAN1A2*, *MGAT1* and *MGAT2* genes expression. Interestingly, studies in T cells homeostasis showed that T cell activation up-regulates the *MGAT5* gene and the GnT-V catalyzes the synthesis of *N*-glycan branching on the TCR,

increasing the binding of galectin 3 (Gal-3) that through the formation of molecular lattices hampers the TCR clustering and consequently the T cell signalling[123,172]. Consistently, our group also showed that $CD4^+$ T cells from UC patients display a deficiency in the *MGAT5* gene, reflecting an altered glycosylation profile associated with the induction of a TCR hyperactivation related with disease severity[145]. This suggests the importance of *N*-glycosylation pathway in the modulation of γδT cells' functions. Since γδT cells present a TCR bearing 6 to 8 *N*-glycosylation sites, we evaluate the impact of this significant post-translational modification in the activity of the γδT cells. We described for the first time a unique and abnormal glycosylation profile of blood γδT cells from SLE patients, suggesting *N*-glycosylation as a possible contributor for γδT cells infiltration in kidney tissue associated with SLE pathogenesis. In these patients, increased expression of high-mannose *N*-glycans in γδT cells is positively associated with the disease severity. Moreover, in γδT cells from SLE patients with lupus nephritis (SLE LN) we observed a reduced expression of β1,6-GlcNAc branched *N*-glycans. Interestingly, the majority of SLE patients without LN (SLE nLN) showed lower values of complex *N*-glycans, despite the higher mean value. Regarding the α-2,6 sialic acid expression in γδT cells, we verified an increase of sialic acid in these cells from SLE patients (LN and nLN). In accordance, a recent study in SLE patients shows an increase of terminal sialylation on T cells associated with less galectin-1 (Gal-1) binding (induce the apoptosis of activated T cells)[156]. The authors propose high sialylation of activated T cells as a mechanism of resistance to apoptosis, that contribute to SLE pathogenesis. Similarly, if the same mechanism occurs in γδT cells, our results suggest that the increased expression of α-2,6 sialic acid in γδT cells might impede the Gal-1 binding, blocking the apoptosis of activated γδT cells and contributing for SLE pathogenesis. As mentioned above, γδT cells are major producers of cytokines, as IL-17 a pro-inflammatory cytokine increased in the lupus nephritis SLE patients that participate in kidney injury[173]. Despite the evidences of IL-17 involvement in SLE pathogenesis, the exact mechanism implicated is still not well elucidated. Interestingly, in our results we showed a lower ratio of L-PHA/GNA in IL-17-producing γδT cells (decrease in β1,6-GlcNAc branched *N*-glycans and an increase of high-mannose *N*-glycans from γδ17T cells) in SLE LN patients. Previous evidences showed that percentage of γδT17 cells is similar between SLE patients and healthy controls[174]. However, we demonstrated that γδ17T cells' glycosylation is altered, which might influence the γδ17T cells activity and function associated with an overexpression of IL-17 in these patients and thus instigating lupus nephritis. With our data, we suggest that the altered glycophenotype present in γδT cells from SLE patients influence γδT cells' function and response and seems to

instigate SLE pathogenesis, proposing β1,6-GlcNAc branched *N*-glycans as immune mediators to control the SLE progression.

Our *in vivo* results have demonstrated different glycosylation profiles associated with activation and function of γδT cells in a tissue-specific manner. As previously described, the *Mgat5*$^{-/-}$ mice (lack of β1,6-GlcNAc branched *N*-glycans) display impaired T cell activation[123]. Accordingly, we have shown a γδT cells' hyperactivation in the kidney, colon and lung from *Mgat5*$^{-/-}$ mice (with 8 weeks- and 15 months-old). Moreover, we showed that γδT cells with increased high-mannose structures have a more hyperactive phenotype, except in γδT cells of lung from aging mice. Accordingly, the γδT cells of spleen from *Mgat5*$^{-/-}$ new-mice exhibited a reduced hyperactivation and lower levels of high-mannose *N*-glycans, as in the WT mice. Since we observed an hyperactivation associated with high-mannose *N*-glycans in γδT cells from peripheric organs, we can speculate that activated γδT cells are infiltrating the tissues and being regulated by glycosylation. Furthermore, we have shown an increased expression of poly-LacNAc structures (LEL) and α-2,6 sialic acid (SNA) in γδT cells that express lower levels of complex *N*-glycans. Accordingly, the lack of branching *N*-glycans in T cells leads to a Golgi's compensatory mechanism that extends the core with poly-LacNAc structures, in order to maintain the homeostasis by the binding between galectins (such as Gal-1 and Gal-3) to poly-LacNac structures[124]. Interestingly, Toscano *et* al. showed that differentiated Th2 express high levels of α-2,6 sialylation at the cell surface, which blocks LacNAc-Gal1 binding and cells are protected from the Gal-1-induced cell death[175]. Taking together, the increase of those glycan-structures in γδT cells from KO mice may be justified by these mechanisms. We can hypothesize that the lack of β1,6-GlcNAc branched *N*-glycans in γδT cells leads to a Golgi's compensatory mechanism that increase the poly-LacNAc structures to maintain the homeostasis. The increase of α-2,6 sialic acid may also occur, suggesting that γδT cells are protect from cell-death through diminished binding of Gal-1 to poly-LacNAc structures. Additionally, the increase of α-2,6 sialic acid might also impede the binding between Gal-3 and poly-LacNAc and consequently lead to impaired γδTCR activation. In this way, glycosylation plays an important role in the modulation of γδT cells' activity and response, reinforcing the impact on autoimmune-like diseases. Moreover, γδT cells of all organs from the *Mgat5*$^{-/-}$ new mice showed an increased expression of intracellular IL-17, when compared with the WT mice. In addition, was observed a slight increase of IFNγ in γδT cells only in the kidney of KO-mice. Conversely, the γδT cells of MLNs (lymphoid tissue) from aging-mice, showed a decreased expression of the intracellular IL-17 and IL-22 in γδT cells. Taking all into consideration, it is suggested that γδT cells showed a specific cytokine response

that is modulated by *N*-glycosylation and dependent on the tissue localization. Additionally, we observed a slight increase in the concentration of IL-17 and IFNγ cytokines in supernatants of renal explants from *Mgat5*$^{-/-}$ aging mice (lack of branched *N*-glycans). Since we also observed an increase of intracellular IL-17 and IFNγ in γδT cells of kidney from *Mgat5*$^{-/-}$-new mice, it suggests that γδT cells may be contributing to the increased production and release of these cytokines in the renal explants. Moreover, other groups demonstrated that in *Mgat5*$^{-/-}$ and/or *Mgat5*$^{+/-}$ mice with induced auto-immune diseases, the supplementation with oral GlcNAc (that promotes *N*-glycans branching) reduces the levels of IFNγ, TNF-α and IL-17 cytokines and delay the disease progression[143,144]. Consistently, SLE-like mouse model deficient in IL-17 were protected from SLE clinical manifestations and autoantibodies formation[176,177]. Altogether, we can hypothesized that γδT cells from the kidney of KO mice exhibit an altered glycoprofile associated with a modulation of γδT cells activity and function, that may instigate renal damage, which is also supported by the presence of kidney autoimmune-like disease in *Mgat5*$^{-/-}$ aging mice[123].

Taken together, we suggest an abnormal *N*-glycosylation as a potential modulator of γδT cells activity and response. Through a glyco-reprogramming of γδT cells, we demonstrated that the lack of complex *N*-glycans in these cells was associated with an autoreactive and pro-inflammatory γδT-phenotype, already suggested in previous results. The chemical inhibition (with KF) of complex *N*-glycans in γδT cells revealed an increased activation compared with the controls. Additionally, we confirmed a decrease of branched *N*-glycans and an increase of high-mannose structures in KF-treated γδT cells. Consistently, previous works in other diseases context demonstrated that the lack of *MGAT5* gene (absence of β1,6-GlcNac branched *N*-glycans) resulted in increased TCR clustering and decreased TCR activation threshold from T cells, leading to T cell hyperactivation and susceptibility to autoimmune diseases[123,142,145]. This is in line with our observations showing that γδT cell's activity can be modulated by *N*-glycosylation and suggesting that when γδTCR loses the branched *N*-glycans it may result in increased γδT cell activity and instigate SLE pathogenesis. Since γδTCR present 6 to 8 potential *N*-glycosylation sites and we confirmed the loss of branched *N*-glycan in the γδTCR of KF-treated γδT cells, these support a potential target for glyco-modulation. Moreover, we further demonstrated that the removal of complex *N*-glycans resulted in increased percentage of γδ17T and γδIFN$_\gamma$T cells and decreased percentage of γδ10T cells, as well as an increase of pro-inflammatory cytokines (IL-17 and IFNγ) and a decrease of anti-inflammatory cytokine (IL-10). This observation are supported by previous evidences from our group, where *ex vivo* GlcNAc supplementation (leads to

enhanced branched *N*-glycosylation) in T cells of the colon from IBD patients, decreased the production of IL-17 and IFNγ[144]. At this point, we demonstrate a clear γδT cells' modulation by *N*-glycans, which is associated with an altered activation status and cytokines response in these cells, converging to an autoreactive and pro-inflammatory γδT cell phenotype. This piece of information pinpoints glyco-modulation as a potential target-therapy in lupus nephritis SLE patients. In order to further validate our results, we reproduced the previous mentioned enzymatic approach in a glycoengineered mouse model, a conditional *Mgat2*$^{-/-}$ mice, that lacks the *Mgat2* gene in *Rag1*$^{+}$T cells (absent in complex *N*-glycans): *Rag1*$^{Cre/+}$*Mgat2*$^{fl/fl}$. We have confirmed an increase of mannose-enriched *N*-glycans (high-mannose and hybrid) associated with an enhanced γδT cells activation from KO mice. In addition, we observed an increase of IL-17 and IFNγ cytokines expression and a decreased of IL-10 cytokine expression in γδT cells. Through an enzymatic and genetic approaches, we demonstrated that γδT cells lacking branched *N*-glycans and enriched in high-mannose structures showed an autoreactive and pro-inflammatory profile.

Finally, we uncovered an important capacity for sensing *N*-glycans by γδT cells. For the first time, we demonstrated the expression of glycan-binding receptors at γδT cells' surface, by targeting the expression of different CTLs. Our *in silico* analysis revealed increased expression of *CLEC7A* (Dectin-1), *CLEC4L* (DC-SIGN) and *CLEC4E* (Mincle) genes, demonstrating that γδT cells might express these receptors at the cell surface. Interestingly, no other studies have been addressing the analysed panel of C-type lectins, except for Dectin-1 that was described in a specific subset of γδT cells[169]. Accordingly, our *in vivo* characterization of γδT cells from SLE patients' blood revealed higher expression of DC-SIGN and MR than the controls, two CTLs that recognize *N*-linked high-mannose and fucose-containing structures[131]. Interestingly, when γδT cells from SLE patients were divided into GNA high or GNA low (increase or decrease of high-mannose *N*-glycans, respectively), we found a prevalence of DC-SIGN in γδT cells with increased levels of high-mannose structures. The opposite was verified in γδT cells with low high-mannose *N*-glycans, which exhibit increase MR expression, demonstrating the association between *N*-glycosylation profile and the expression of γδT cell' CTLs, especially the DC-SIGN. Moreover, γδT cells from *Mgat5*$^{-/-}$ aging mice also display increased expression of MR in the peripheral organs. In accordance, an early interruption of the *N*-glycosylation pathway (conditional *Mgat2*$^{-/-}$ mice) demonstrates a noticeable increase expression of MR in γδT cells. The increased expression of these CTLs on the cell surface may confers a better γδT cells' capacity to sense abnormal *N*-glycosylation from the environment, such as DAMPs and PAMPs[178–181]. This "awake" status of γδT

cells may be contributing to their hyperactivation and the perpetuation of a pro-inflammatory profile of these cells in autoimmunity. Accordingly, also human KF-treated γδT cells presented increased expression of DC-SIGN. These mice and human glycoengineered models resemble the findings observed in the SLE patients, where γδT cells with deficient β1,6-GlcNAc branched *N*-glycans revealed an overexpression of DC-SIGN. Curiously, some researchers' findings revealed increase expression of DC-SIGN during monocyte-dendritic cell differentiation or in human liver endothelial cells principally due to increased expression of IL-4, a cytokine that can also be secreted by γδT cells subsets, that is known to be increased in blood of SLE patients, like others cytokines (IL-17, IL-18, IL-12)[182–186]. Thus, it can be hypothesized that the low complex *N*-glycans in γδT cells lead to an abnormal cytokine imbalance, which may increase the expression of DC-SIGN in γδT cells from SLE patients. Additionally, the altered CTLs expression at γδT cell surface may increase the recognition capacity of γδT cells to identify altered self-mannose *N*-glycans as danger antigens and consequently instigate and perpetuate SLE etiopathogenesis.

In summary, in this thesis work we have demonstrated, for the first time, the role of glycosylation alterations in γδT cells function and response. We demonstrated an abnormal overexpression of high-mannose *N*-glycans in γδT cells from SLE patients, associated with an increased infiltration of these cells in patients' kidney. The similar was observed in autoimmune-susceptible mice, where γδT cells are infiltrating the colon and kidney from those mice. Moreover, we showed a tissue-specific glycophenotype of γδT cells, potentially associated with disease pathogenesis. Also, we demonstrate that the ablation of branched *N*-glycans combined with increased high-mannose *N*-glycans leads to γδT cells hyperactivation and a more pro-inflammatory γδT cells' profile. Finally, but equally relevant, we showed for the first time, that γδT cells express at least two CTLs at the cell surface and that an altered γδT cells' glycophenotype results in increased expression of DC-SIGN and MR. Altogether, our study shows that γδT cells exhibit an altered glycosignature associated with an autoreactive and pro-inflammatory phenotype, revealing a potential new mechanism in SLE etiopathogenesis.

Conclusion and future perspectives

In this master thesis, we have demonstrated for the first time the relevance of glycosylation alterations in γδT cells activity and function in both SLE patients and in immune-mediated mouse model. Specifically, an increased high-mannose *N*-glycans in γδT cells associated with enhanced γδT cells activity and infiltration in kidney of SLE patients and in other organs from autoimmune-susceptible mouse model. As so, we propose that an increased expression of high-mannose *N*-glycans in γδT cells is a key determinant that regulate the activation of these cells and leads to an autoreactive and pro-inflammatory γδT cells' immune response, that may instigate SLE pathogenesis. Importantly, we further showed, for the first time, the expression of two CTLs at γδT cells' surface, in which DC-SIGN is particularly upregulated in high-mannosylated γδT cells. This phenotype might be associated with an increased recognition of high-mannose glycans by γδT cells as potential danger antigens, constituting a trigger in SLE development. The proposed model of this thesis is demonstrated in Figure 27.

Further analyses are needed to validate the exact role of γδT cells' glycoprofile alterations in SLE pathogenesis. Firstly, we need to consolidate our results, by increasing the number of SLE cases and mice samples analysed. In addition, we aim to characterize a transgenic lupus mouse model, that lack γδT cells and branched *N*-glycans, to assess the impact of glycosylation in these γδT cell deficient mice.

Altogether, we herein propose a new mechanism in SLE immunopathogenesis mediated by an altered glycosylation profile of γδT cells. This will constitute a potential targeted specific therapeutic approach in SLE, by increasing the branched *N*-glycans of γδT cells from SLE patients to control their activity and function.

The results presented in this thesis resulted in two scientific abstracts and one publication in which I am co-author:

- **Abstract accepted for publication** - *Annals of the Rheumatic Diseases* - "Changes in cellular glycosylation as a key factor in the immunopathogenesis of Systemic Lupus Erythematosus" - I. Alves, V. Pinto, B. Santos-Pereira, A. Campar, J. R. Vizcaino, F. Carneiro, C. Vasconcelos, A. Marinho, S. Pinho - *Annals of the Rheumatic Diseases* 2020; 79:1361-1362.
- **Abstract accepted for poster** - Virtual Society for Glycobiology Annual Meeting 2020 - "Protein mannosylation as a diagnostic and prognostic biomarker of lupus nephritis: an unusual glycan-neoepitope in Systemic Lupus Erythematosus" – I. Alves, B. Santos-Pereira, M. Vicente, H. Dalebout, A. Campar, S. Santos, M. Thepaut, F.

Fieschi, S. Strahl, F. Boyaval, R. Vizcaíno, R. Silva, S. Holst-Bernal, L. .Santos, M. Wuhrer, A. Marinho, B. Heijs, S. Pinho.

- **Scientific publication** - "Protein mannosylation as a diagnostic and prognostic biomarker of lupus nephritis: an unusual glycan-neoepitope in Systemic Lupus Erythematosus" – I. Alves, B. Santos-Pereira, M. Vicente, H. Dalebout, A. Campar, S. Santos, M. Thepaut, F. Fieschi, S. Strahl, F. Boyaval, R. Vizcaíno, R. Silva, S. Holst-Bernal, L. .Santos, M. Wuhrer, A. Marinho, B. Heijs, S. Pinho – under submission.

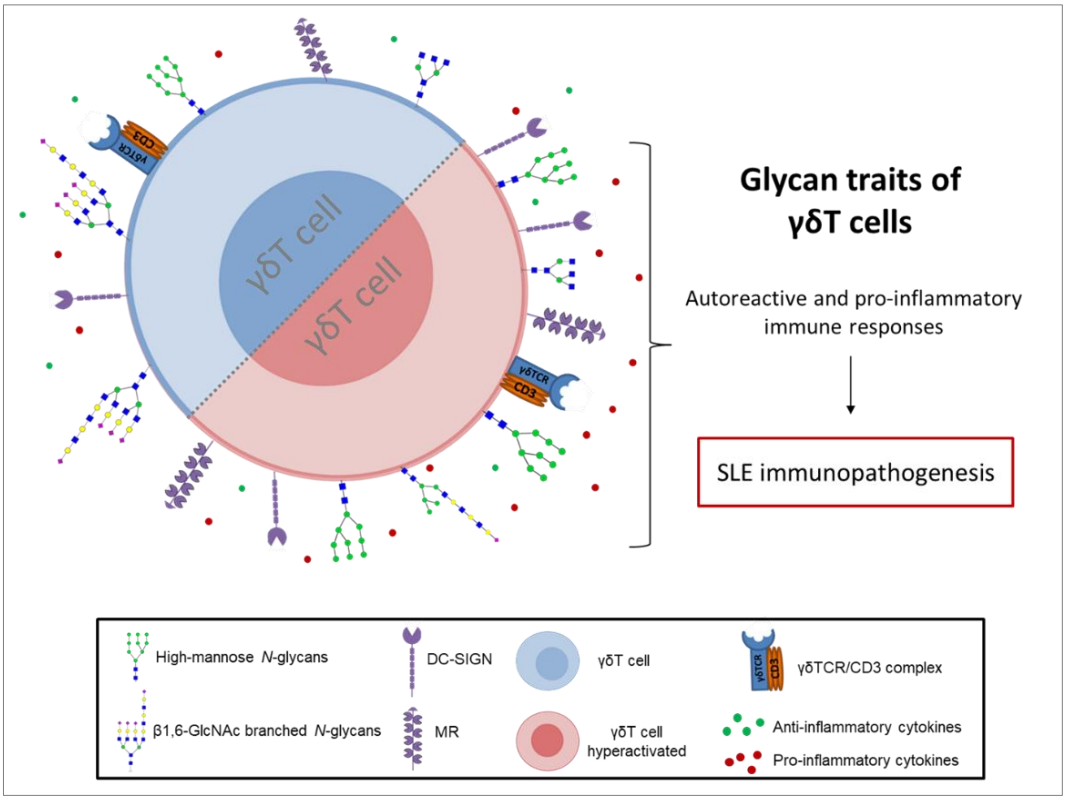

Figure 27 – Proposed model. The γδT cells expressing balanced type of *N*-glycans (high-mannose, complex and hybrid N-glycans) showed a controlled activity and response (blue). Although, dysregulations in *N*-glycosylation pathway leads to a decreased on complex *N*-glycans and increased of high-mannose *N*-glycans (red). This results in impaired γδT cells activation and increased pro-inflammatory cytokines release, as well as overexpression of DC-SIGN and MR at cell surface. Converging to an autoreactive and pro-inflammatory immune response by γδT cells, which may instigate SLE immunopathogenesis.

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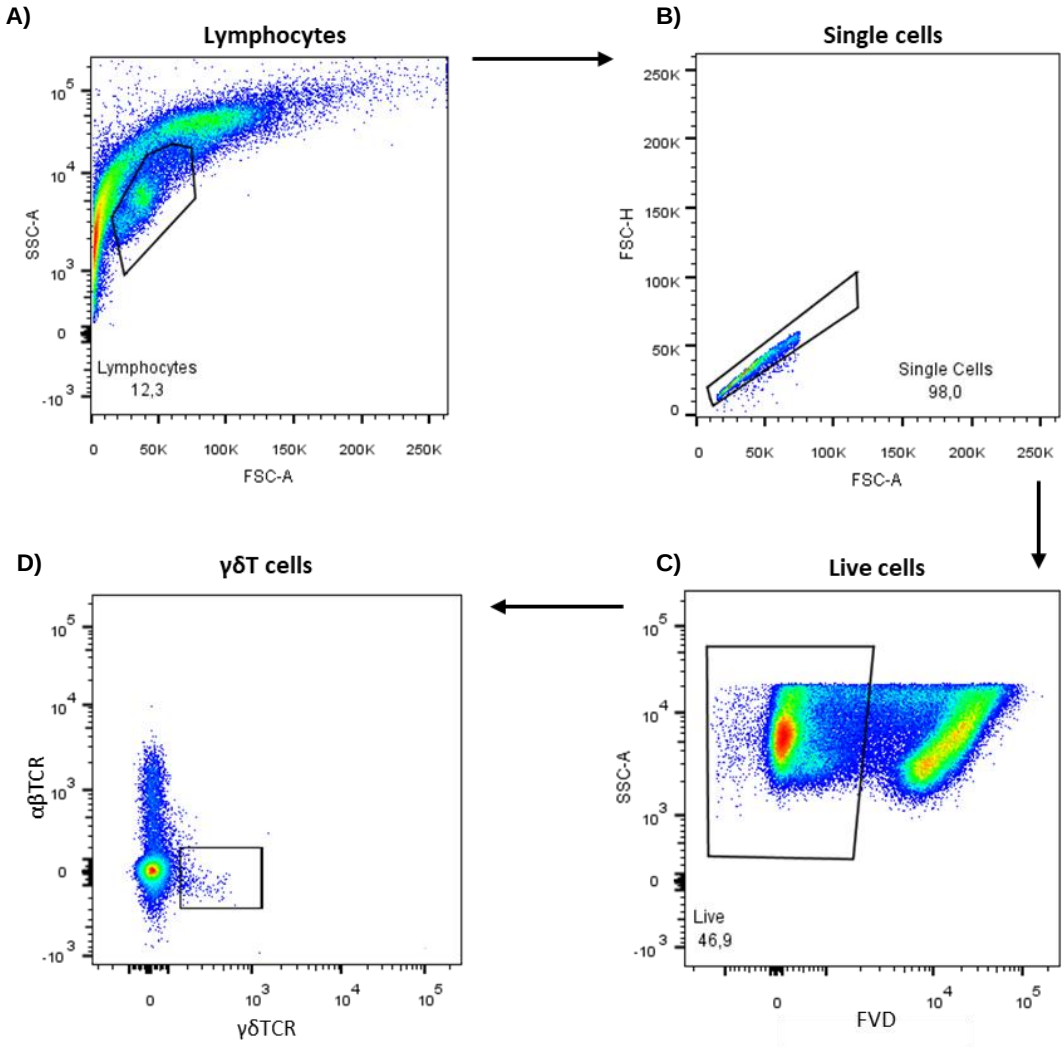
Appendix



Supplementary figure 1 – Representative example of the gating strategy to define γδT cells in flow cytometry analysis. A) Total lymphocytes were gated on a forward scatter (FSC)/side scatter (SSC) plot based on size and granularity (complexity). **B)** The single cells were gated based on forward scatter height (FSC-H) and forward scatter area (FSC-A) and the doublet cells eliminated. **C)** Alive cells were gated by non-incorporation of the fixable viability dye (FVD). **D)** The γδT cells were identified and gated based on the positive expression for γδTCR and negative expression for αβTCR ($\gamma\delta TCR^+ \alpha\beta TCR^-$). The data files were analysed with FlowJo software and the results were represented in the GraphPad Prism 7 and expressed as the median fluorescent intensity (MFI) or the MFI fold-change of each lectin/antigen expression.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